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? s oxygen
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? type s25/full/all

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S6     29492  DS
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S19      187  S17 AND S18
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S21        0  S14 AND S3
S22       14  S14 AND RECOMBINANT?
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S26        0  DIERYCK
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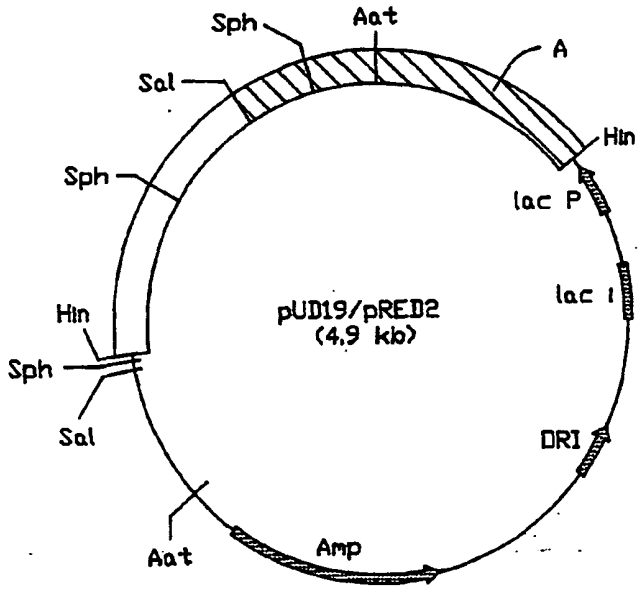
(54) Title: ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF A CLONED HEMOGLOBIN GENE



(57) Abstract

The invention related to a gene promoter/regulator (Region A in Fig. 1) which is useful in subjecting expression of protein or polypeptides in a host cell to selective regulation by external control. In particular, this regulation may be accomplished to different extends by the level of dissolved oxygen, presence of cAMP-CAP, and/or a complex nitrogen source in the culture or environment. The invention also relates to a method of improving production of proteins in a host by coproduction of hemoglobin in the host.

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ENHANCEMENT OF CELL GROWTH BY EXPRESSION OF A CLONED HEMOGLOBIN GENE

This is a continuation-in-part of Serial No. 342,451, filed January 24, 1989 as PCT application No. PCT US88-03745, which is a continuation-in-part of Serial No. 113,014 filed October 23, 1987 and Serial No. 151,526, filed February 2, 1988.

TECHNICAL FIELD

This invention relates to the production of proteins and polypeptides, and to production of oxygen-binding proteins, particularly hemoglobins, and to enhancement of the growth and product synthesis characteristics of aerobic organisms in environments with sufficient as well as reduced or low levels of oxygen.

This invention relates generally to the use of recombinant DNA technology to direct or otherwise control gene expression in cultured cells, and more particularly, to methods and materials useful in subjecting the transcription and translation of DNA sequences to selective regulation by external control.

BACKGROUND ART

Globins such as hemoglobin and myoglobin are hemecontaining oxygen carriers. By reversibly binding to oxygen in the presence of high oxygen concentrations and releasing it in regions or at times of low concentrations, these proteins considerably enhance the oxygen uptake rate of multicellular organisms over that allowed by mere passive diffusion. In unicellular organisms it is generally believed that the oxygen uptake rate is principally limited by the rate of transfer of dissolved oxygen in the environment or growth medium to the exterior cell surface. However, closer examination of cell structure reveals several potential diffusional barriers between environmental oxygen and the cytochromes where the oxygen finally undergoes reaction. For example, in gram negative bacteria, where the cytochromes are attached to the inside of the plasma membrane, the diffusing oxygen needs to cross transport barriers such as the cell wall, the outer membrane, the periplasmic space and the inner membrane before accepting electrons from metabolic reactions. In unicellular eucaryotes, where oxidative phosphorylation takes place in the mitochondria, there are further diffusional resistances. Small neutral molecules like oxygen are assumed to passively diffuse across these barriers; however, these barriers make a non-trivial contribution to the overall resistance to mass transfer to the actual reaction site and thus could be of significance under oxygen-limited conditions.

Physiological effects on growth due to depletion in dissolved oxygen levels has been demonstrated in the case of several organisms, including Escherichia coli, Saccharomyces cerevisiae, Pseudomonas strains, and Alcaligenes eutrophus. In E. coli for example,

which has a very high affinity cytochrome, changes in dissolved oxygen tension leads to differential regulation of terminal oxidases, resulting in a decrease in the number of protons expelled per NADH molecule oxidized during aerobic respiration and, consequently, a possible adverse change in the stoichiometry of ATP biosynthesis. (Kranz et al., Journal of Bacteriology 158:1191-1194, 1984; Ingraham et al., Growth of the bacterial cell, Sinauer Associates, Inc. 1983, p. 147, both specifically incorporated herein.)

In addition to the respiratory oxygen requirement of aerobic organisms, oxygen-binding proteins have other potential applications as well, including, for example, the enhancement of particular oxidative transformations such as steroid conversions, vinegar production, biological waste treatment or enzymatic degradations, and in some steps in brewing or making distilled and fermented foods and beverages.

The filamentous bacterium, Vitreoscilla, a member of the Beggiatoa family, is a strict aerobe that is found in oxygen-poor environments such as stagnant ponds and decaying vegetable matter. Growth of the bacterium under hypoxic conditions results in a several-fold induction of synthesis of a homodimeric soluble heme protein (subunit MW 15,775) (Boerman et al., Control of heme content in Vitreoscilla by oxygen, Journal of General Applied Microbiology 28:35-42, 1982) which has a remarkable spectral (Webster, et al., Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochrome o purified from Vitreoscilla, Journal of Biological Chemistry 249:4257-4260, 1974), structural (Wakabayashi, et al., Primary sequences of a dimeric bacterial hemoglobin from Vitreoscilla, Nature

322:481-483, 1986), and kinetic (Orii, et al., Photodissociation of oxygenated cytochrome o(xs) (Vitreoscilla) and kinetic studies of reassociation, Journal of Biol gical Chemistry 261:2978-2986, 1986) homology with eucaryotic hemoglobins, and which is probably a true bacterial hemoglobin.

This protein was previously thought to be a cytochrome o, and it has been suggested to function in oxygen storage. However, biochemical discrepancies (Webster, et al., Oxygenated cytochrome o, Journal of Biological Chemistry 252:1834-1836, 1977) as well as the subsequent discovery of the true membrane-bound cytochromes o and d (DeMaio, et al., Spectral evidence for the existence of a second cytochrome o in whole cells of Vitreoscilla, Journal of Biological Chemistry 258:13768-13771, 1983; Webster et al., Federation Proceeding 44:678, 1985) led to further investigations of its spectral properties (Choc et al., Oxygenated intermediate and carbonyl species of cytochrome o (Vitreoscilla), Journal of Biological Chemistry 257: 865-869, 1982; Orii et al., supra.) and the eventual determination of its probable amino acid sequences and partial homology with known hemoglobin sequences (23).

Although these articles disclose the conservation of most features characteristic of eucaryotic hemoglobins, and discuss, to some extent, the role or potential role it probably plays in oxygen utilization, none of these researchers had previously been able to isolate a portable DNA sequence capable of directing intracellular production of this bacterial hemoglobin or to create a recombinant-DNA method for its production. Additionally, there has been no published proof of any oxygen transport or other kinetic function for this protein in

Vitreoscilla, or any suggestion in the literature of any benefit from the introduction of a bacterial hemoglobin in heterologous organisms. Moreover, there has been no suggestion that such an oxygen-binding protein would have a far-reaching range of applications.

Surprisingly, the present inventors have discovered a portable DNA sequence capable of directing the recombinant-DNA synthesis of a bacterial hemoglobin. The hemoglobin of the present invention, prepared by the recombinant-DNA methods set forth herein, will enable increased research into the growth of organisms in oxygen-poor environments. In addition, the oxygen-binding proteins of the present invention are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes, and for binding and separating oxygen from other fluids or gases. Furthermore, the oxygen-binding proteins of this invention are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. The proteins of this invention are also useful as selective markers in recombinant-DNA work, and have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing, and particular oxidative reactions and transformations.

This invention also relates to certain DNA sequences which usually precede a gene in a DNA polymer and which provide a site for initiation of the transcription of that gene into mRNA. These are referred to as "promoter" sequences. Other DNA or RNA sequences, also usually but not necessarily

"upstream" of a structural gene, bind proteins that determine the frequency or rate of transcription and/or translation initiation. These ther sequences, including attenuators, enhancers, operators and the like, are referred to as "regulator" sequences. Thus, sequences which operate to determine whether the transcription and eventual expression of a gene will take place are collectively referred to as "promoter/regulator" DNA sequences.

The promoter/regulator sequences of genes are susceptible to enormous structural and functional variation, and in general, serve to regulate gene transcription in response to chemical and, sometimes, physical environmental conditions in and around the cell. Several generalized models for the action of promoter/regulator operation in gene transcription have been proposed. One model utilizes a repressor gene and a regulator sequence or operator sequence near the promoter of another gene. According to this model, transcription of the repressor sequence results in expression of a repressor protein which selectively binds to the operator sequence to effectively preclude transcription of the selected gene. An environmental signal, such as the increased concentration of a chemical acted upon by the protein product of the gene in question, may operatively inactivate the repressor protein, blocking its ability to bind to the operator sequence in a way which would interrupt transcription of the gene. Increased concentrations of a substrate could be seen as operating to induce synthesis of the protein which catalyzes its breakdown.

Another generalized model of operation of promoter/regulator sequences in the regulation of gene transcription suggests formation of an initially

inactive form of repressor protein by the repressor DNA sequence. Such inactive form could not bind to an operator DNA sequence and disrupt selected gene transcription until it is combined with some other substance present in the cell, such as a compound which is the product of a reaction catalyzed by the protein coded for by the selected gene. Increased concentrations of such a reaction product in the cell would thus operate to repress the potential overproduction of proteins responsible for the product's synthesis. In these examples, the regulator protein functions to inhibit transcription. Other regulatory proteins have been described which potentiate or activate transcription of specific DNA sequences. Thus, there can be both positive and negative control proteins and corresponding regulatory DNA sequences.

Regulation of gene expression can also occur at the level of translation. For example, a regulator molecule could bind to a particular site on the messenger RNA, thus inhibiting or blocking translation.

Much of the genetic engineering activity to date has been oriented toward stably incorporating foreign DNA into cells, to provide not only a source of multiple copies of selected genes, but the large scale transcription and expression of commercially significant gene products.

The lactose ("lac") promoter/operator systems have been commonly used, for they are very controllable through the mode of action of the operator. When the operator is repressed, the DNA dependent RNA polymerase is completely prevented from binding and initiating transcription, thus effectively blocking

promoter operability. This system can be derepressed by induction following the addition of a known inducer, such as isopropyl-beta-D-thiogalactoside (IPTG). The inducer causes the repressor protein to fall away so the RNA polymerase can function.

Cells transformed with plasmids carrying the lac promoter/operator system can be permitted to grow up to maximal density while in the repressed state through the omission of an inducer, such as IPTG, from the media. When a high level of cell density is achieved, the system can be derepressed by addition of inducer. The promoter is then free to initiate transcription and thus obtain expression of the gene products at yields commensurate with the promoter strength. However, certain of these inducible promoter systems are relatively weak and commercial or research productions using such systems do not urge the cell to generate maximum output.

In response to the need for microbial expression vehicles capable of producing desired products in higher yield, the tryptophan ("trp") promoter/ operator system has become widely used. This system is one of several known systems with at least three times the strength of the lac promoter. However, it has the disadvantage of less promoter control. The trp promoter is not inducible in the way the lac promoter is, namely, the bound repressor is not removed by induction. Instead, the system operates on a sort of feedback loop as described above. A system was devised whereby the attenuator region of the trp promoter/operator system was removed, with the resultant transformed cells being grown in tryptophan-rich media. This provided sufficient tryptophan to essentially completely repress the operator so that cell growth could proceed

uninhibited by premature expression of any desired foreign prot ins. When the culture reached appropriat growth levels, no additional tryptophan was supplied, resulting in mild tryptophan limitation, and, accordingly, derepression of the promoter with resultant expression of the desired protein gene insert. In application, this system has several disadvantages. For example, it is necessary to maintain high levels of tryptophan in the growth media to completely repress the promoter, and to permit the medium to become completely exhausted of tryptophan following full growth of the culture.

A hybrid system has been developed from the tryptophan and lactose promoter, wherein both promoters can be repressed by the lac repressor and both can be derepressed with IPTG. See De Boer et al., The tac promoter: A functional hybrid derived from the trp and lac promoters, Proc. Natl. Acad. Sci. USA, 80: 21-25, 1983. This system shares a disadvantage with the two discussed above, namely the required introduction of additional agents to a normal growth medium.

Another regulator/promoter system commonly used for expression of cloned proteins in E. coli is based on the P_L promoter system from phage lambda. See Bernard and Helsinki, Methods in Enzymology, 68:482-492, 1979; Use of Lambda Phage Promoter PL to Promote Gene Expression In Hybrid Plasmid Cloning Vehicles. Induction of this promoter requires increase of culture temperature from 30°C to 42°C. This system has the disadvantages of suboptimal growth rates at 30°C prior to induction and upsetting of cell metabolism by the temperature shift. Temperature shift effects on metabolism are discussed, for example, by Neidhart, et. al., The Genetics and

Regulation Of Heat-Shock Proteins, Annual Reviews of Genetics, 18:295-329, 1984.

There has been a need in the art for an economical, simple, highly controllable and efficient promoter/regulator system for subjecting the transcription of DNA sequences to selective regulation by external control at constant temperature. The present inventors have discovered such an expression system, which can switch from low to very high expression activity upon reduction of dissolved oxygen concentration in the medium. This reduction in dissolved oxygen (DO) level is easily implemented at high cell densities without the need for addition of any chemical to the growth medium to induce gene expression.

In addition, other modes of regulation independent of or in conjunction with control of DO have been discovered.

DISCLOSURE OF THE INVENTION

The present invention relates to portable DNA sequences capable of directing intracellular production of proteins and polypeptides, including oxygen-binding proteins, particularly hemoglobins. The present invention also relates to vectors containing these portable DNA sequences.

One object of the present invention is to provide an active (i.e., oxygen-transporting form) bacterial hemoglobin protein, which can be produced in sufficient quantities and purities to provide economical pharmaceutical, laboratory or industrial compositions which possess oxygen-binding activity.

An additional object of the present invention is to provid a recombinant-DNA method for the production of these oxygen-binding proteins. To facilitate the recombinant-DNA synthesis of these oxygen-binding proteins, it is a further object of the present invention to provide portable DNA sequences capable of directing intracellular production of oxygen-binding proteins. It is also an object of the present invention to provide cloning vectors containing these portable sequences. These vectors are capable of being used in recombinant systems to enhance the growth characteristics of organisms, and to produce useful quantities of oxygen-binding proteins. Augmented by intracellular synthesis of oxygen-binding proteins, product formation may also be enhanced.

The present invention also provides novel methods and materials for subjecting DNA sequences of living microorganisms to external regulation which is dependent upon availability of oxygen in the environment. Particularly, it relates to promoter/regulators, a recombinant-DNA method of producing same, and to portable DNA sequences capable of directing the translation and transcription initiation and control of the expression of desired gene products.

Thus, another object of the present invention is to provide for the control of expression of any selected chromosomal or extrachromosomal gene or DNA sequence through the incorporation of a promoter/regulator DNA sequence which is functionally responsive to environmental variations in the concentration of oxygen. The invention is thus broadly applicable to a variety of aerobic or slightly aerobic procedures for controlling genetic processes, ranging from the

alteration of existing regulation of endogenous genes in prokaryotic and eucaryotic cells to securing selectiv , differential regulation of expression of selected exogenous or foreign genes stably incorporated in host cells.

Still another object of the present invention is to provide a promoter/regulator as described above, which can be produced in sufficient quantities and purities to provide their economical pharmaceutical, laboratory or industrial use.

Additional objects and advantages of the invention will be set forth in part in the description which follows, and in part will be obvious from the description or may be learned from practice of the invention. The objects and advantages may be realized and attained by means of the instrumentalities and combination particularly pointed out in the appended claims.

To achieve the objects and in accordance with the purposes of the present invention, promoter/ regulators are also set forth. To further achieve the objects and in accordance with the purposes of the present invention, as embodied and broadly described herein, portable DNA sequences for these promoter/regulators are provided. Particularly preferred promoter/regulator DNA sequences for use in the practice of the present invention are derived from the filamentous bacterium Vitreoscilla. Portable nucleotide sequences are provided for these promoter/regulators. The portable sequences may be either synthetic sequences or restriction fragments ("natural" DNA sequences).

To facilitate identification and isolation of natural DNA sequences for use in the present invention, the inventors have developed a Vitreoscilla genomic library. This library contains the genetic information capabl of directing a cell to synthesize the hemoglobin of the present invention. Other natural DNA sequences which may be used in the recombinant DNA methods set forth herein may be isolated from other genomic libraries.

Additionally, portable DNA sequences useful in the processes of the present invention may be synthetically created. These synthetic DNA sequences may be prepared by polynucleotide synthesis and sequencing techniques known to those of ordinary skill in the art.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, a recombinant-DNA method is disclosed which results in manufacture by a host cell or microorganism of oxygen-binding proteins or other cloned proteins or polypeptides using the portable DNA sequences referred to above.

Additionally, to achieve the objects and in accordance with the purposes of the present invention, recombinant-DNA methods are disclosed which subject to external control the translation and transcription of gene products by a host cell or microorganism using the portable DNA sequences referred to above.

Processes of the invention include methods for subjecting the expression of a selected DNA sequence in a living cell or virus to regulation by oxygen level through the site-specific insertion of

promoter/regulator DNA sequences responsive thereto. Also disclosed are improvements in prior methods for securing expression of a selected "foreign" or exogenous sequence in a host microorganism wherein the DNA sequence is stably incorporated as chromosomal or extrachromosomal constituent of the host. Such improvements comprise fusing to the selected DNA sequence a promoter/regulator DNA sequence capable of selectively promoting or inhibiting expression of the selected DNA in response to variations in environmental concentration of oxygen. It has now been found that expression of oxygen-binding proteins, particularly in oxygen-poor environments, can improve intracellular production of other proteins. Thus, a host cell containing a first expression vector (extrachromosomal or chromosomal) for expression of a designated protein may be modified to enhance production of that protein by introducing into the host a second expression vector (which may or may not be part of the first expression vector) for expression of an oxygen-binding protein. In an oxygen-poor environment, the second vector improves the cell productivity, and thus the production of the designated protein.

It has further been found that there are modes of regulating the activity of the Vitreoscilla hemoglobin promoter/regulator other than by environmental oxygen concentration (DO). During the exponential growth phase of a host cell culture, it has been found that addition of a complex nitrogen source, such as yeast extract, to the culture allows suppression of the Vitreoscilla hemoglobin promoter/regulator independently of environmental oxygen concentration. It has also been found that the presence of the cAMP-CAP complex is important to the activity of the Vitreoscilla hemoglobin

promoter/regulator, thus manipulation of the activity crp gene in a host cell is another method of regulation. For example, using a crp mutant host, by direct addition of CAP t the culture the activity of the Vitreoscilla promoter/regulator may be regulated.

It is understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive of the invention, as claimed.

The accompanying drawing, which is incorporated in and constitutes a part of this specification, illustrates one embodiment of the invention and, together with the description, serves to explain the principles of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a partial restriction map of the plasmid pUC19/pRED2.

BEST MODES FOR CARRYING OUT THE INVENTION

Reference will now be made in detail to the presently preferred embodiments of the invention, which, together with the drawing and the following examples, serve to explain the principles of the invention.

It must be understood that the present inventors have prepared an oxygen-binding protein and its natural promoter/regulator by recombinant DNA methods. While some methods for the production and use of these recombinant products are described below, the end use of these products alone is within the scope of the present invention.

As noted above, the present invention relates in part to portable DNA sequences capable of directing

intracellular production of oxygen-binding proteins in a variety of host cells and host microorganisms. "Portable DNA sequence" in this context is intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence. For purposes of this specification, "oxygen-binding protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the amino acid sequence, and which may or may not include post-translational modifications. It is contemplated that such post-translational modifications include, for example, association with a heme prosthetic group. It is further intended that the term "oxygen-binding protein" refers to either the form of the protein as would be excreted from a cell or as it may be present in the cell from which it was not excreted.

In a preferred embodiment, the portable DNA sequences are capable of directing intracellular production of hemoglobin. In a particularly preferred embodiment, the portable DNA sequences are capable of directing intracellular production of a hemoglobin biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "biologically equivalent", as used herein, it is meant that a protein, produced using a portable DNA sequence of the present invention, is capable of binding oxygen in the same fashion, but not necessarily to the same degree, as the homodimeric soluble heme protein (subunit MW 15,775) isolable from Vitreoscilla.

As noted above, the present invention also relates in part to portable DNA sequences which contain

promoter/regulators which are capable of directing intracellular expression f endogenous or exogenous gene products, in a variety of host cells and h st microorganisms. "Portable DNA sequence" and "promoter/regulator" in this context are intended to refer either to a synthetically produced nucleotide sequence or to a restriction fragment of a naturally occurring DNA sequence.

The portable DNA sequences of the present invention may also include DNA sequences downstream from a promoter/regulator which code for at least one foreign protein. For purposes of this specificatIon, "foreign protein" is intended to mean a protein with a primary structure as defined by the codons present in the deoxyribonucleic acid sequence which directs intracellular production of the corresponding amino acid sequence, and which may or may not include post-translational modifications. It is further intended that the term "foreign protein" refers to either the form of the protein as it would be excreted from a cell or as it may be present in the cell from which it was not excreted.

While the precise mechanism of regulation is not certain, the promoter/regulator is capable of directing intracellular production of hemoglobin and/or other operatively fused gene products upon a drop in oxygen available to the host cell. Also, expression of oxygen-binding proteins, particularly in oxygen-poor environments, can improve intracellular production of other proteins. The activity of the Vitreoscilla hemoglobin promoter/regulator may be regulated other than by environmental oxygen concentration. During the exponential growth phase of a host cell culture, the addition of a complex nitrogen source, such as yeast

extract, to the cultur allows suppression of the Vitreoscilla hem globin promoter/regulator independently of environmental oxygen concentration. Since the cAMP-CAP complex is important to the activity of the Vitreoscilla hemoglobin promoter/regulator, manipulation of the activity of the crp gene in a host cell is another method of regulation.

In a particularly preferred embodiment, the promoter/regulator contains transcription and translation initiation and control sequences substantially equivalent to those for directing intracellular production of a hemoglobin protein biologically equivalent to that previously isolated from the filamentous bacterium, Vitreoscilla. By "substantially equivalent", as used herein, is meant that a promoter/regulator operates to express a downstream gene product upon reduction of the level of oxygen available to the host cell below some critical value.

It is of course intended that the promoter/regulators of the present invention may control and initiate transcription and translation of an unlimited number of endogenous and/or exogenous foreign proteins.

A first preferred portable DNA sequence for the promoter/regulators of the present invention contains at least a portion of the following nucleotide sequence, which reads 5' to 3' and includes the translation initiation sequence ATG (underlined) and some of the nucleotide sequence of the Vitreoscilla structural gene (also underlined):

Hin:

AAGCTTAACG GACCCTGGGG TTAAAAGTAT TTGAGTTTTG
ATGTGGATTA AGTTTTAAGA 60

GGCAATAAAG ATTATAATAA GTGCTGCTAC ACCATACTGA
TGTATGGCAA AACCATAATA 120

ATGAACTTAA GGAAGACCCT CATGTTAGAC CAGCAAACCA
TTAACATCAT CAAAGCCACT 180

GTTCCTGTAT TGAAGGAGCA TGGCGTTACC ATTACCACGA
CTTTTTATAA AAACTTGTTT 240

GCCAAACACC CTGAAGTACG TCCTTTGTTT GATATGGGTC
GCCAAGAATC TTTGGAGCAG 300

CCTAAGGCTT TGGCGATGAC GGTATTGGCG GCAGCGCAAA
ACATTGAAAA TTTGCCAGCT 360

ATTTTGCCTG CGGTCAAAAA AATTGCAGTC AAACATTGTC
AAGCAGGCGR GGCAGCAGCG 420

CATTATCCGA TTGTCGGTCA AGAATTGTTG GGTGCGATTA
AAGAAGTATT GGGCGATGCC 480

GCAACCGATG ACATTTTGGA CGCGTGGGGC AAGGCTTATG
GCGTGATTGC AGATGTGTTT 540

ATTCAAGTGG AAGCAGATTT GTACGCTCAA GCGGTTGAAT
AAAGTTTCAG GCCGCTTTCA 600

GGACATAAAA AACGCACCAT AAGGTGGTCT TTTTACGTCT
GATATTTACA CAGCAGCAGT 660

TTGGCTGTTG GCCAAAACTT GGGACAAATA TTGCCCTGTG
TAAGAGCCCG CCGTTGCTGC 720

GACGTCTTCA GGTGTGCCTT GGCAT 745

Th nucleotide bases represented by the above abbreviations are as follows: A = Adenine, G = Guanine, C = Cytosine, and T = Thymine.

The above sequence exhibits homology with certain sequences which are highly conserved in a variety of promoter/regulators. Using conventional numbering, with the underlining showing the homology in the above sequence to the consensus sequence, the -120 consensus sequence or Pribnow box sequence is TATAAT(A/G). The -35 consensus sequence is TTGACA, and the consensus Shine-Dalgarno sequence is AGGAGGTXXX(XX)ATG.

In a preferred embodiment, the above sequence is operatively fused with at least a portion of a downstream sequence of nucleotides which code for at least a portion of the Vitreoscilla hemoglobin protein which contains at least a portion of the following amino acid sequence:

5 10
Met-Leu-Asp-Gln-Gln-Thr-Ile-Asn-Ile-Ile-

15 20
Lys-Ala-Thr-Val-Pro-Val-Leu-Lys-Glu-His-

25 30
Gly-Val-Thr-Ile-Thr-Thr-Thr-Phe-Tyr-Lys-

35 40
Asn-Leu-Phe-Ala-Lys-His-Pro-Glu-Val-Arg-

45 50
Pro-Leu-Phe-Asp-Met-Gly-Arg-Gln-Glu-Ser-

55 60
Leu-Glu-Gln-Pro-Lys-Ala-Leu-Ala-Met-Thr-

65 70
Val-Leu-Ala-Ala-Ala-Gln-Asn-Ile-Glu-Asn-

75 80
Leu-Pro-Ala-Ile-Leu-Pro-Ala-Val-Lys-Lys-

85 90
Ile-Ala-Val-Lys-His-Cys-Gln-Ala-Gly-Val-

95 100
Ala-Ala-Ala-His-Tyr-Pro-Ile-Val-Gly-Gln-

105 110
Glu-Leu-Leu-Gly-Ala-Ile-Lys-Glu-Val-Leu-

115 120
Gly-Asp-Ala-Ala-Thr-Asp-Asp-Ile-Leu-Asp-

125 130
Ala-Trp-Gly-Lys-Ala-Tyr-Gly-Val-Ile-Ala-

135 140
Asp-Val-Phe-Ile-Gln-Val-Glu-Ala-Asp-Leu-

145 150
Tyr-Ala-Gln-Ala-Val-Glu

This amino acid sequence is disclosed in Wakabayashi et al., supra, Nature 322:483, 1986. It is presently believed that the protein purified and prepared through the practice of this invention will exhibit a homology of over 80% with this sequence. The protein of this invention has been observed to enhance

functioning of a cell in low oxygen environments (Khosla and Bailey, unpublished results).

The amino acids represented by the foregoing abbreviations are as follows:

Amino Acid	3-Letter Symbol
Glycine	Gly
Alanine	Ala
Valine	Val
Leucine	Leu
Isoleucine	Ile
Arginine	Arg
Lysine	Lys
Glutamic acid	Glu
Aspartic acid	Asp
Glutamine	Gln
Asparagine	Asn
Threonine	Thr
Serine	Ser
Cysteine	Cys
Methionine	Met
Phenylalanine	Phe
Tyrosine	Tyr
Tryptophan	Trp
Proline	Pro
Histidine	His

It must be borne in mind in the practice of the present invention that the alteration of some amino acids in a protein sequence may not affect the fundamental properties of the protein. Therefore, it is also contemplated that other portable DNA sequences, both those capable of directing intracellular production of identical amino acid sequences and those capable of directing intracellular production of analogous amino acid sequences which also possess oxygen-binding activity, are included within the ambit of the present invention.

It must also be borne in mind in the practice of the present invention that the alteration of some

nucleotide bases in a DNA sequence may not affect the fundamental properties of the coding sequence. Therefore, it is also contemplated that other analogous portable DNA promoter/regulator sequences which are operable through changes in oxygen level are included within the ambit of the present invention.

It is contemplated that some of these analogous amino acid sequences will be substantially homologous to native Vitreoscilla hemoglobin while other amino acid sequences, capable of functioning as oxygen-binding proteins, will not exhibit substantial homology to native Vitreoscilla hemoglobin. By "substantial homology" as used herein, is meant a degree of homology to native Vitreoscilla hemoglobin in excess of 50%, preferably in excess of 80%.

Similarly, it is contemplated that some of these analogous DNA sequences will be substantially homologous to the sequence set forth above, while other DNA sequences, capable of functioning as the promoter/regulator described above, will not exhibit substantial homology to the sequence outlined above.

As noted above, the portable DNA sequences of the present invention may be synthetically created, by hand or with automated apparatus. It is believed that the means for synthetic creation of these polynucleotide sequences are generally known to one of ordinary skill in the art, particularly in light of the teachings contained herein. As examples of the current state of the art relating to polynucleotide synthesis, one is directed to Maniatis et al., Molecular Cloning--A Laboratory Manual, Cold Spring Harbor Laboratory (1984), and Horvath et al., An Automated DNA Synthesizer Employing Deoxynucleoside

3'-Phosphoramidit s, Methods in Enzymology 154:313-326, 1987, hereby incorporated by reference.

Additionally, the portable DNA sequence may be a fragment of a natural sequence, i.e., a fragment of a polynucleotide which occurred in nature and which has been cloned and expressed for the first time by the present inventors. In one embodiment, the portable DNA sequence is a restriction fragment isolated from a genomic library. In this preferred embodiment, the genomic library is created from the bacterium Vitreoscilla. In other alternative embodiments, the portable DNA sequence is isolated from other genomic and cDNA libraries.

While it is envisioned that the portable DNA sequences of this invention may desirably be inserted directly into the host chromosome, the present invention also provides a series of vectors, each containing at least one of the portable DNA sequences described herein. It is contemplated that additional copies of the portable DNA sequence may be included in a single vector to increase a host cell's ability to produce large quantities of the desired oxygen-binding protein. It is also envisioned that other desirable DNA sequences may also be included in the vectors of this invention. Further, the invention may be practiced through the use of multiple vectors, with additional copies of at least one of the portable DNA sequences of this invention and perhaps other desirable DNA sequences.

In addition, the cloning vectors within the scope of the present invention may contain supplemental nucleotide sequences preceding or subsequent to the portable promoter/regulator and/or DNA sequence. These supplemental sequences are those that will not

adversely interfer with transcription of the portable promoter/regulator and/or any fused DNA sequence and will, in some instances, enhance transcription, translation, posttranslational processing, or the ability of the primary amino acid structure of the resultant gene product to assume an active form.

A preferred vector of the present invention is set forth in Figure 1. This vector, pUC19/pRED2, contains the preferred nucleotide sequence which codes for the amino acids set forth above. Vector pUC19/pRED2 cells are on deposit in the American Type Culture Collection ("ATCC") in Rockville, Maryland under Accession No. 67536.

A preferred nucleotide sequence encoding the Vitreoscilla hemoglobin protein and adjacent Vitreoscilla sequences described above is identified in Figure 1 as region A. The above nucleotide sequence reads counterclockwise through region A of Figure 1. Plasmid pUC19/pRED2 may also contain supplemental nucleotide sequences preceding and subsequent to the preferred DNA sequence in region A, such as terminators, enhancers, attenuators and the like. For proteins to be exported from the intracellular space, at least one leader sequence and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA may be included within the scope of this invention.

In a preferred embodiment, cloning vectors containing and capable of expressing the portable DNA sequence of the present invention contain various operational elements in addition to or instead of the promoter/regulator disclosed and claimed herein.

These "operational elements" may include at least one promoter, at least one sequence that acts as expression regulator, and at least one terminator codon, at least one leader sequence, and any other DNA sequences necessary or preferred for appropriate transcription and subsequent translation of the vector DNA.

Additional embodiments of the present invention are envisioned as employing other known or currently undiscovered vectors which would contain one or more of the portable DNA sequences described herein. In particular, it is preferred that these vectors have some or all of the following characteristics: (1) possess a minimal number of host-organism sequences; (2) be stable in the desired host; (3) be capable of being present in a high copy number in the desired host; (4) possess a regulatable promoter; and (5) have at least one DNA sequence coding for a selectable trait present on a portion of the plasmid separate from that where the portable DNA sequence will be inserted. Alteration of vectors to meet the above criteria are easily performed by those of ordinary skill in the art in light of the available literature and the teachings herein. It is to be understood that additional cloning vectors may now exist or will be discovered which have the above-identified properties and are therefore suitable for use in the present invention and these vectors are also contemplated as being within the scope of this invention.

As set forth in Example 1, an E. coli vector system is a preferred embodiment. Various cloning vehicles are required for the range of host cells and organisms suitable for insertion of the portable DNA sequences of the present invention, as set forth

below. In light f the available literature, choice of such a cloning vehicle, if necessary, is within the ordinary skill in the art.

Additional bacterial hosts are suitable, including, without limitation: bacteria such as members of the genera Bacillus, Pseudomonas, Alcaligenes, Streptococcus, Lactobacillus, Methylophilus, Xanthomonas, Corynebacterium, Brevibacterium, Acetobacter, and Streptomyces.

Examples of suitable eucaryotic host microorganisms would include fungi, yeasts such as Saccharomyces and Candida, and molds such as Asnergillus, Pennicillim and Cephalosporium.

It is envisioned that the scope of this invention would cover expression systems in eucaryotic microorganisms and host cultured cells derived from multicellular organisms, including animals, insects and plants, which are grown in the presence of oxygen. The promoter/regulator of the present invention is especially useful in a host which switches from low to very high expression activity upon reduction of dissolved oxygen concentration in the medium. Such expression systems need not be derived from Vitreoscilla.

Various vector systems will be suitable for these and other desirable hosts, including plasmids, viruses and bacteriophages. The following, noninclusive list of cloning vectors is believed to set forth vectors which can easily be altered to meet the above criteria and are therefore preferred for use in the present invention. Such alterations are easily performed by those of ordinary skill in the art in

light of the availabl literature and the teaching herein.

For example, many selectable cloning vectors have been characterized for use in E. coli, including pUC8, pUC9, pBR322, pGW7, placI^q, and pDP8, Maniatis et al., supra. A bifunctional vector that replicates in E. coli and can also be used in Streptomyces is pKC462a. Suitable vectors for use in Bacillus include: pUB110, pSA0501, pSA2100, pBD6, pBD8, and pT127, Ganesan and Hock, eds., Genetics and Biotechnology of Bacilli, Academic Press 1984. In Pseudomonas, RSF1010, Pms149, pKT209, and RK2 are suitable; some of these vectors are useful in a wide range of gram-negative bacteria including Agrobacterium and Xanthomonas. For Saccharomyces, it is possible to use YEp24, Y1p5, and YRp17, Botstein and Davis, Molecular Biology of the Yeast Saccharomyces (Strathern et al., eds.), Cold Spring Harbor Laboratory, 1982. In mammalian systems retrovirus vectors such as those derived from SV40 are typically used.

Synthesis and/or isolation of necessary and desired component parts of cloning vectors, and their assembly is believed to be within the duties and tasks performed by those with ordinary skill in the art and, as such, are capable of being performed without undue experimentation.

In construction of the cloning vectors of the present invention, it should additionally be noted that multiple copies of the promoter/regulator with any fused gene sequences and/or of the portable DNA sequence coding for the oxygen-binding protein and its attendant operational elements as necessary may be inserted into each vector. In such an embodiment,

the host organism would produce greater amounts per vector of th desired cloned protein. The number of multiple copies of the DNA sequence which may be inserted into the vector is limited only by the ability of the resultant vector, due to its size, to be transferred into and replicated and expressed in an appropriate host.

Additionally, it is preferred that the cloning vector contain a selectable marker, such as a drug resistance marker or other marker which causes expression of a selectable trait by the host. In a particularly preferred embodiment of the present invention, the gene for ampicillin resistance is included in vector pUC19/pRED2. Such a drug resistance or other selectable marker is intended in part to facilitate in the selection of transformants. Additionally, the presence of such a selectable marker on the cloning vector may be of use in keeping contaminating microorganisms from multiplying in the culture medium. In this embodiment, such a pure culture of the transformed host organisms would be obtained by culturing the organisms under conditions which require the induced phenotype for survival.

It is noted that the portable DNA sequence of the present invention may themselves be used as a selectable marker, in that they provide enhanced growth characteristics in low oxygen circumstances, and also engender an easily visible reddish tint in the host cells.

The promoter/regulators of this invention are capable of controlling expression of proteins or, thereby, of controlling synthesis of metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic

manipulation. This would include heterol g us proteins--either intracellular or extracellular--as well as biopolymers such as polysaccharide materials, simpler metabolites such as amino acids and nucleotides, antibiotics and other chemicals produced by living cells or cellular biocatalysts.

The oxygen-binding proteins of the present invention, prepared by the recombinant-DNA methods set forth herein, will enable increased research into the growth of organisms in oxygen-poor environments. In addition, the oxygen-binding proteins of the present invention are useful in enhancing oxygen supply to cells or in other oxygen-utilizing processes (Adlercreutz et al., Biocatalyst in Organic Synthesis, Symposium of the Working Party on Immobilized Biocatalysts of the European Federation of Biotechnology, Abstracts, p.18, 1985), and for binding and separating oxygen from other fluids or gases (Bonaventura et al., Underwater Life Support Based on Immobilized Oxygen Carriers, Applied Biochemistry and Biotechnology 9: 65-80, 1984). Furthermore, the oxygen-binding proteins of this invention are capable of increasing production of cells, or of proteins or metabolites normally made by a cell, or of natural or unnatural metabolites and proteins expressed in a cell via genetic manipulation. This would, as described above, include heterologous proteins, biopolymers, simpler metabolites, antibiotics, and other chemicals produced by living cells or cellular biocatalysts.

The protein products of this invention also have applications as diverse as enhancing certain oxygen-requiring steps in fermentation, enzymatic degradation, toxic chemical waste treatment, brewing

and particular oxidative reactions and transformations such as steroid conversions.

This invention also relates to a recombinant-DNA method for the production of oxygen-binding proteins. Generally, this method includes:

(a) preparing a portable DNA sequence capable of directing a host cell or microorganism to produce a protein having oxygen-binding activity;

(b) transferring the portable DNA sequence directly into the host, or cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell or microorganism, such vector containing operational elements for the portable DNA sequence;

(c) transferring the vector containing the portable DNA sequence and operational elements into a host cell or microorganism capable of expressing the oxygen-binding protein;

(d) culturing the host microorganism under conditions appropriate for replication and propagation of the vector and/or expression of the protein; and

(e) in either order:

(i) harvesting protein; and

(ii) permitting the protein to assume an active structure whereby it possesses oxygen-binding activity.

In this method, the portable DNA sequences are those synthetic or naturally-occurring polynucleotides described above. In a preferred embodiment, the

portable DNA sequence codes for at least a portion of the Vitreoscilla hemoglobin protein described above.

This invention also relates to a recombinant-DNA method for the use of thes promoter/regulators. Generally, this method provides a process for subjecting the expression of a selected DNA sequence to external control under given environmental conditions which comprises the steps of:

(a) providing at least one selected isolated structural gene that is transcriptionally and/or translationally responsive to a Vitreoscilla hemoglobin promoter/regulator DNA sequence under the given environmental conditions; and

(b) operatively fusing the selected structural gene with said promoter/regulator DNA sequence.

It is envisioned that the portable DNA sequences may be inserted directly into the host chromosome, or alternatively may utilize a vector cloning system. The vectors contemplated as being useful in the present method are those described above. In a preferred embodiment, the cloning vector pUC19/pRED2 is used in the disclosed method.

A vector thus obtained may then be transferred into the appropriate host cell or organism. It is believed that any microorganism having the ability to take up exogenous DNA and express those genes and attendant operational elements may be chosen. Particular hosts which may be preferable for use in this invention include those described above. Methods for transfer of vectors into hosts are within the ordinary skill in the art. For ultimate expression in certain microorganisms such as yeast, it may be desirable that the cloning vector be first transferred into another microorganism such as

Escherichia coli, where the vector would be allowed t replicate and, from which the vector would be obtained and purified after amplification, and then transferred into the yeast for ultimate expression of the oxygen-binding protein.

The host cells or microorganisms are cultured under conditions appropriate for the expression of the oxygen-binding protein. These conditions are generally specific for the host organism, and are readily determined by one of ordinary skill in the art, in light of the published literature regarding the growth conditions for such organisms.

In one embodiment, conditions necessary for the regulation of the expression of the DNA sequence, dependent upon any operational elements inserted into or present in the vector, would be in effect at the transformation and culturing stages. The cells are grown to a high density in the presence of appropriate regulatory conditions which inhibit the expression of the DNA sequence. When optimal cell density is approached, the environmental conditions are altered to those appropriate for the expression of the portable DNA sequence. It is thus contemplated that the production of a cloned protein will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant cloned protein product would be harvested, if desired, at some time after the regulatory conditions necessary for its expression were induced.

Where the operational elements used are in the promoter/regulator sequence of this invention, these conditions are as follows. The cells are grown to a high density in the presence of appropriate levels of oxygen which inhibit the expression of the DNA

sequence. When optimal cell density is approached, the environmental oxygen level is altered to a lower value appropriate for the expression of the portable DNA sequence. Levels from less than in a 1% oxygen-saturated solution to oxygen saturated are within the scope of this invention. It is thus contemplated that the production of any desired fused product will occur in a time span subsequent to the growth of the host cells to near optimal density, and that the resultant product would be harvested, if desired, at some time after the oxygen level necessary for its expression were reached.

If harvesting of the oxygen-binding protein products of the present invention is desired, it may be done prior or subsequent to purification and prior or subsequent to assumption of an active structure.

It is currently believed that some percentage of the oxygen-binding proteins of the present invention will assume their proper, active structure upon expression in the host cell or organism. If desired, the oxygen-binding protein may be transported across a cell membrane. This will generally occur if DNA coding for an appropriate leader sequence has been linked to the DNA coding for the recombinant protein. The structures of numerous signal peptides have been published. It is envisioned that these leader sequences, included in or added to at least some portion of the portable DNA as necessary, will direct intracellular production of a fusion protein which will be transported through the cell membrane and will have the leader sequence cleaved upon release from the cell.

Additional uses of the oxygen-binding proteins of the present invention are envisioned. The purified

proteins and/or the wh le cells and/or extracts of the cells of the present invention themselves may be used to bind to oxygen or proteins and thus could function somewhat as erythrocytes.

The present invention may also be used as a method for transporting and enhancing oxygen supply to cells or in other oxygen-utilizing processes by delivering the oxygen-binding proteins -- isolated in lysates and crude cell preparations, purified from extracts, in synthetic sequences, or in whole cells containing the proteins -- where desired. It is envisioned that the protein products of the present invention could valuably be added to media for culturing cells and thereby enhance the transport of oxygen.

It is also envisioned that the proteins of the present invention may be used for binding and separating of oxygen from fluids such as seawater and from other gases.

It is understood that application of the teachings of the present invention to a specific problem or environment will be within the capabilities of one having ordinary skill in the art in light of teachings contained herein. Examples of the products of the present invention and representative processes for their isolation, use and manufacture appear below.

INDUSTRIAL APPLICABILITY

The products and processes of the present invention find usefulness in a range of medical, laboratory and industrial applications. The invention provides metabolically engineered cells with enhanced growth characteristics for increasing production of various

proteins or metabolites by those cells. The invention further provides a method for subjecting expression of a certain DNA sequence to external control under given environmental conditions. Also provided are recombinant-DNA fusion gene products, expression vectors, and nucleotide base sequences for the practice of the invention. The products and processes of the present invention find applications in a range of aerobic processes, such as manufacture of cloned proteins and synthesis of metabolites, chemical production by fermentation, enzymatic degradation, waste treatment, brewing and a range of oxidative reactions.

EXAMPLES

EXAMPLE 1 - CLONING AND EXPRESSION OF HEMOGLOBIN FROM VITREOSCILLA IN ESCHERICHIA COLI.

Materials and Methods. Vitreoscilla sp. (Murray strain no. 389) was obtained from Dr. Webster (Department of Biology, Illinois institute of Technology, Chicago, Illinois 60616, USA), and grown in a medium containing 1.5% yeast extract, 1.5% peptone, and 0.02% sodium acetate (pH 8.0 with NaOH).

E. coli JM101 were obtained from the laboratory of Dr. Simon (Division of Biology, California Institute of Technology, Pasadena, California 91125, USA), and grown in L broth containing 1% Bactotryptone, 0.5% yeast extract and 1% sodium chloride.

Plasmid pUC19 (Yanisch-Perron et al., Improved M13 phase cloning vectors and host strains: nucleotide sequences of m13mp18 and nUC19 vectors, Gene 33: 103-109, 1985) packaging kits were purchased from Pharmacia. All restriction enzymes, T4

polynucleotide kinase and T4 ligase were from New England Biolabs or Bethesda Research Laboratories. Calf intestine alkaline ph sphatase was from Pharmacia. Mixed oligonucleotide probes were synthesized with an Applied Biosystems synthesizer. Kodak XAR5 x-ray film was used for autoradiography. Geneclean kits were purchased from Bio101. All other chemicals were of analytical grade.

Vitreoscilla genomic DNA was isolated according to the protocol of Silhavy et al., Experiments with gene fusions, Cold Spring Harbor Laboratory (1984), specifically incorporated herein. HindIII-digested Vitreoscilla DNA was ligated into the phosphatased HindIII site of pUC19 and transformed into JM101. Recombinant colonies and plaques were transferred on nitrocellulose filters as described in Maniatis, et al., Molecular cloning--a laboratory manual, Cold Spring Harbor Laboratory (1982) and specifically incorporated herein. Rapid plasmid isolation from recombinant colonies were done according to Silhavy et al, supra. Digested fragments of plasmid DNA or fractions of genomic DNA were isolated from agarose gels using Geneclean kits. E. coli cells were transformed by the CaC12 method of Silhavy et al., supra. Plasmid uptake was induced by heat-shocking chilled competent cells at 37°C for 5 minutes.

For Southern hybridizations the reagents suggested in Dupont catalog No. NEF-976, Protocols for electrophoretic and capillary transfer of DNA and RNA DNA and RNA hybridization and DNA and RNA rehybridization (1985), specifically incorporated herein, were used, whereas for colony and plaque hybridizations those described in Maniatis et al., supra, were used. Filters were prehybridized at 45-50 C for 2-4 hours and hybridized at 30°C for 20-24

hours. 200 picomoles oligonucleotide kinased with 200 microCi (32_p)ATP (sp. act. 7000 Ci/mmol) were used as probe. Filters were washed in 2 X SSC, 0.1% SDS at room temperature (3 X 5 minutes) and at 46°C (for the C-terminal probe) and 50°C (for the N-terminal probe) prior to autoradiography.

SDS-polyacrylamide gel electrophoresis was done according to standard protocols, Laemmli, Cleavage of structural proteins during the assembly of the head of bacteriophage T4, Nature 227:680-685, 1970, specifically incorporated herein, with a 12.5% resolving gel. Protein in the gel was visualized by the silver staining method of Merril et al., Ultrasensitive stain for proteins in polyacrylamide gels shows regional variation in cerebrospinal fluid proteins, Science 211:1437-1438, 1983.

Results. Three sets of mixed oligonucleotide probes were synthesized which had a predicted homology to two domains in the hemoglobin gene, one N-terminal and one C-terminal. A pUC19-HindIII library of Vitreoscilla DNA was test-plated on rich plates with ampicillin, X-gal and IPTG. More than 70% of the colonies were probable recombinants, as estimated by visual inspection. About 10,000 colonies were then screened. Three positives were identified. Because of the high density of colonies on the plate, these, along with their immediate clones from each group, were assayed by rapid isolation and HindIII digestion. One of these, pRED1, had three inserted fragments including a 2.2kb one. Subsequent digestion of this plasmid with various endonucleases and Southern hybridization of the resulting DNA bands did confirm that the 2.2kb band did indeed contain the entire hemoglobin gene, since no HindIII sites

are expected to exist upstream or downstream of the regions spanned by the oligomeric probes.

The HindIII fragment from pRED1 that contained th hemoglobin structural gene was purifi d and reinserted by standard protocols into pUC19 in both orientations (pRED2 and pRED3). E. coli cells containing plasmids pRED1, pRED2, pRED3 and pUC19 as well as Vitreoscilla cells were grown to stationary phase and cell extracts were assayed on an SDS-polyacrylamide gel for the existence of the hemoglobin polypeptide. The hemoglobin was expressed as a major cellular protein in all recombinant cells. Since both plasmids pRED2 and pRED3 express about equal amounts of this polypeptide, it is presently believed that the gene is probably expressed from its natural promoter in E. coli.

The restriction map of plasmid pRED2 is shown in Figure 1.

EXAMPLE 2 - COMPLETE NUCLEOTIDE SEQUENCE.

Determination of the sequence of the relevant region of the fragment isolated from the Vitreoscilla genomic library was accomplished as follows:

The HindIII-SphI fragment from plasmid pRED2 which contains the structural gene and adjacent sequences was subcloned into pUC19 (purchased from Bethesda Research Labs) to obtain plasmid pRED4. An MluI site was identified, by restriction mapping the resulting plasmid, which breaks up the HindIII-SphI insert into two fragments which were individually sequenced using conventional protocols (Maxam and Gilbert, Sequencing end-labeled DNA with base-specific chemical cleavages, Methods in Enzymology 65: 499-560, 1980; Iverson and Dervan, Adenine specific DNA chemical

sequencing reaction, Nuclear Acids Research 15: 7823-7830, 1987).

The nucleotide sequence of the important portion of the HindIII-SphI fragment is as listed below. It includes a putative E. coli promoter, ribosome binding site, the complete VHb structural gene (start and stop codons are underlined) and a putative E. coli transcription terminator (Khosla and Bailey, The Vitreoscilla hemoglobin gene: molecular cloning nucleotide sequence and genetic expression in Escherichia coli, Mol. & Gen. Genet., in press).

AAGCTTAACG GACGCTGGGG TTAAAAGTAT TTGAGTTTTG
ATGTGGATTA AGTTTTAAGA 60

GGCAATAAAG ATTATAATAA GTGCTGCTAC ACCATACTGA
TGTATGGCAA AACCATAATA 120

ATGAACTTAA GGAAGACCCT CATGTTAGAC CAGCAAACCA
TTAACATCAT CAAAGCCACT 180

GTTCCTGTAT TGAAGGAGCA TGGCGTTACC ATTACCACGA
CTTTTTATAA AAACTTGTTT 240

GCCAAACACC CTGAAGTACG TCCTTTGTTT GATATGGGTC
GCCAAGAATC TTTGGAGCAG 300

CCTAAGGCTT TGGCGATGAC GGTATTGGCG GCAGCGCAAA
ACATTGAAAA TTTGCCAGCT 360

ATTTTGCCTG CGGTCAAAAA AATTGCAGTC AAAOGATTGTC
AAGCAGGCGR GGCAGCAGCG 420

CATTATCCGA TTGTCGGTCA AGAATTGTTG GGTGCGATTA
AAGAAGTATT GGGCGATGCC 480

GCAACCGATG ACATTTTGGA CGCGTGGGGC AAGGCTTATG
GCGTGATTGC AGATGTGTTT 540

ATTCAAGTGG AAGCAGATTT GTACGCTCAA GCGGTTGAAT
AAAGTTTCAG GCCGCTTTCA 600

GGACATAAAA AACGCACCAT AAGGTGGTCT TTTTACGTCT
GATATTTACA CAGCAGCAGT 660

TTGGCTGTTG GCCAAAACTT GGGACAAATA TTGCCCTGTG
TAAGAGCCCG CCGTTGCTGC 720

GACGTCTTCA GGTGTGCCTT GCCAT 745

EXAMPLE 3 - GROWTH ENHANCEMENT IN E. COLI WITH pRED2: SHAKE FLASK CULTURES.

In this Example, the growth behavior of E. coli cells producing active Vitreoscilla hemoglobin was compared to that of control strains grown under identical conditions. The following strains were studied: (1) JM101:pRED2; (2) JM101:pUC9; and (3) JM101. Plasmids pUC9 and pUC19 are essentially identical except for a difference in one restriction site unrelated to the insert or to any of the functional properties of the plasmid.

Experimental protocol: Cells were grown at 37°C in a complex medium containing 1% (W/V) bactotryptone, 0.5% (W/V) yeast extract, 0.5% (W/V) NaCl, 0.3% (W/V) K_2HPO_4 and 0.1% (W/V) KH_2PO4 (pH 7.0). Plasmid-containing cells were grown in the presence of 100 mg/L ampicillin. In each case the shake-flask was inoculated with a 1% (V/V) dose of concentrated nutrient broth containing 430 g/L glucose, 5 g/L yeast extract, 110 g/L $(NH_4)_2SO_4$, 8 g/L $MgSO_4 \cdot 7H_{20}$, 0.27 g/L $FeCl_3 \cdot 6H_{20}$, 0.02 g/L $ZnCl_2 \cdot 4H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.02 g/L $Na^2MoO_4 \cdot 2H_2O$, 0.01 g/L

$CuSO_4 \cdot 5H_2O$, 0.005 g/L H_3BO_3, 0.1% (V/V) conc. HCl, 4.2 mg/L riboflavin, 54 mg/L pantothenic acid, 60 mg/L folic acid. This formulation has been successfully used on a previous occasion to grow stationary cells to a high density in a fedbatch mode. The cells were then allowed to grow further until stationary phase was reached again. Optical density was measured at 600 nm on a Bausch & Lomb Spectronic 21 spectrophotometer. Dry weights were measured by spinning 10 mL samples at 4°C, washing once with distilled water and subsequently drying the resuspended sample at 100°C overnight. The heme content of the cells was assayed according to the method of Lamba & Webster (Lamba & Webster, Effect of growth conditions on yield and heme content of Vitreoscilla, Journal of Bacteriology 142: 169-173, 1980), and the hemoglobin activity was measured by the method of Webster & Liu (Webster and Liu, Reduced nicotinamide adenine dinucleotide cytochrome o reductase associated with cytochromic o purified from Vitreoscilla, Journal of Biological Chemistry, 249: 4257-4260, 1974).

Results. The growth properties, heme content and hemoglobin activity of the three strains are documented in the Table below.

	JM101:pRED2	JM101:pUC9	JM101
1. OD_{600} before nutrient replenishment	0.937	0.737	0.945
2. OD_{600}	1.230	0.880	0.985
3. max. attained dry wt.	1.5g/L	0.85g/L	1g/L
4. relative heme content	5.5	1	**
5. relative hemoglobin activity	5	1	**
6. specific growth rate*	0.04/h	0.01/h	0.009/h

*mean value following additional feeding of shake-flasks as described above

**not assayed

EXAMPLE 4

GROWTH ENHANCEMENT OF E. COLI WITH pRED2.

A typical high-cell density fermentation is of a fed-batch type. The optimal rate of nutrient addition, and consequently the productivity, is ultimately limited by the rate at which cells can aerobically catabolize the carbon source without generating growth-inhibitory metabolites such as acetate and lactate (Zabriskie and Arcuri, Factors influencing productivity of fermentations employing recombinant microorganisms, Enzyme and Microbial Technology 8: 706-717, 1986; Tsai et al, The effect of organic nitrogen and glucose on the productivity of recombinant insulin-like growth factor in high cell density Escherichia coli fermentations, Journal of Industrial Microbiology 2: 181-187, 1987). In this experiment, we compare the growth properties of the recombinant strain (JM101:pRED2) expressing Vitreoscilla hemoglobin with similar plasmid-containing (JM101:pUC9) and plasmid-free (JM101)

strains under typical fed-batch fermentation conditions.

Materials and Methods:

Cells were grown in a New Brunswick Microferm fermentor at 37+0.5°C and a pH of 7±0.05 with an initial working volume of 2.5 L. A constant air-flow rate of 4.5 L/min and agitator speed of 300 rpm were maintained throughout each run. Silicone anti foam AF60 was used to control foaming. The batch medium and feed medium 1 listed in Table 2 in Tsai et al., supra. were used. Growth following inoculation was in batch mode. After batch stationary phase was reached, continuous feeding was initiated using feed medium 1 at a flow rate of 10 mL/hr. For plasmid-containing cells, 100 mg/L ampicillin was used. In all cases, the dissolved oxygen (DO) levels remained fairly constant around 5% of air saturation for most of the run except during the early log phase and towards batch stationary phase.

Results:

The growth parameters measured for the three strains are listed below. Batch stationary phase refers to conditions before continuous feeding was started.

	JM101	JM101:pUC9	JM101:pRED2
Batch log-phase growth rate (h^{-1})	0.95	0.73	0.95
Batch stationary-phase dry cell mass (g/L)	2.6	1.6	2.6
Fed-batch log-phase growth rate (h^{-1})	0.056	0.033	0.066
Final dry cell mass (g/L)	5.8	2.8	5.9

Further, the respiratory behavior of JM101:pRED2 was improved compared to the control strains at low DO levels, as observ d in a Gilson respirometer.

Conclusion:

Cells containing Vitreoscilla hemoglobin grow faster and to higher densities than comparable plasmid-containing controls.

EXAMPLE 5

EXPRESSION OF VITREOSCILLA HEMOGLOBIN (VHb) IN E. COLI UNDER THE REGULATION OF OTHER PROMOTERS

In Examples 1, 3, and 4 above, the expression of hemoglobin is under the regulation of its native oxygen-regulated promoter. Hence, it is not possible to modulate independently the dissolved oxygen concentration (DO) and the intracellular VHb level. In order to overcome this, the inventors attempted to express this protein under the control of other regulatable promoters which are functional in E. coli, such as trp (Russell and Bennett, Construction and analysis of in vitro activity of E. coli promoter hybrids and promoter mutants that alter the -35 to -10 spacing, Gene 20: 231-243, 1982) and tac (deBoer et al., The tac promoter: a functional hybrid derived from the trp and lac promoters, Proc. Natl. Acad. Sci. USA 80: 21-25, 1983).

Materials and Methods:

Plasmid pRED4 (see Example 2) was linearized with HindIII and treated with exonuclease Ba131 to generate 5'end deletions in the HindIII-SphI VHb fragment (Maniatis et al., supra). After digestion with SphI, the resulting VHb fragments were cloned into HindIII-SphI digested pUC19. The positions of the deleted end-points were identified by sequencing (protocol similar to that in Example 2).

trp and tac promoters and the chloramphenicol acetyl transferase gene (CAT) were purchased from Pharmacia, Inc. Oligonucleotides were synthesized at California Institute of Techn logy using an Applied Biosystems DNA synthesizer. All DNA nzymes were obtained from vendors.

The functional assay for the VHb gene product is as described in Webster and Liu, supra.

Cells were pelleted at 4°C and resuspended in 100 mM Tris (pH 7.5), 50 mM NaCl. This cell suspension was sonicated at 75 W for 3 min. on ice. After spinning in at 12,000 g for 10 min., the supernatant was collected and assayed for VHb. Total protein content was estimated using the Bradford assay kit from BioRad Inc. VHb activity is reported as delta-A_{419436}/mg total protein.

Results:

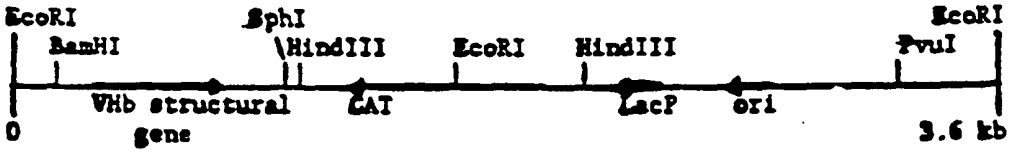
One of the deletions, pRED302, mapped 2 base-pairs upstream of the ATG start codon for the VHb structural gene. This deletion was used for further work. The EcoRI/BamHI trp promoter cartridge was cloned upstream of the truncated VHb fragment. The following ribosome binding site was synthesized:

5' GATCCCGGGTCTAGAGGA 3'
GGCCCAGATCTCCT

and inserted between the BamHI and nuclease-blunted XbaI sites to give rise to a trp promoter-controlled VHb expression system. The CAT gene (Alton and Vapnek, Nucleotide sequence analysis of the chloramphenicol resistance transposon Tn9, Nature 282: 864-869, 1979) was inserted downstream and under the control of the lac promoter available on

this pUC19-based plasmid. This gene product can be conveniently assayed (Neumann et al, Novel rapid assay for chloramphenicol acetyltransferase gene expression, BioTechniques 5: 444-447, 1987) and henc serv s as a useful reporter. Finally, the B-lactamase gene on this pUC19-based plasmid was deleted by digestion and religation with PuS. The purpose of this step is to eliminate the presence of a plasmid-encoded periplasmic protein. The plasmid thus obtained was called pHbCAT and was transformed into JM101. As a control, the CAT gene was cloned downstream and under the control of the lac promoter in pUC19. The B-lactamase gene was identically deleted. This plasmid was called pCAT. The restriction maps and the anticipated sequence of relevant regions of these two plasmids are shown below.

pHbCAT (3.6 kb)



EcoRi

5' GAATT CCCCT GTTGA CAATT AATCA TCCAA CTAGT TAACT AGTAC

BamHI

GCAGC TTGGC TGCAG GTCGA CGGAT CCCGG GRCTA GAGGA AGTCT

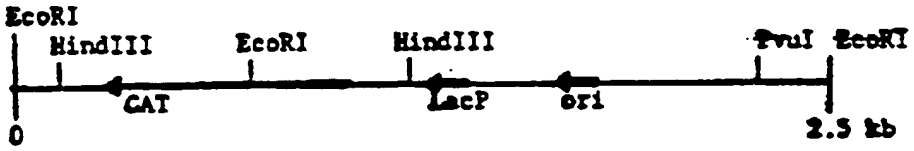
Start codon of VHb

CATGT TAGAC ..... (same as in Example 3 up to SphI site)

The sequence of the region spanning between EcoRI and the start of the VHb structural gene is shown above.

It includes th trp promoter and a synthetic ribosome binding site.

pCAT (2.5 kb)



The effect of tryptophan (repressor) and indole acrylic acid (gratuitous inducer) on VHb levels in JM101/pHbCAT are shown in the Table below. In these experiments, cells were grown to mid-log in minimal medium containing 3 g/L glycerol, 3 g/L Casamino acids, and the appropriate amount of indole-acrylic acid or tryptophan.

Host: Plasmid	Tryptophan (mg/L)	Indole-acrylic acid (mg/L)	Specific Hb Activity*
JM101:pCAT	--	--	3.4×10^{-3}
JM101:pHbCAT	20	--	6.2×10^{-3}
"	4	--	18.3×10^{-3}
"	--	--	31.5×10^{-3}
"	--	1	29.8×10^{-3}
"	--	2.53	6.5×10^{-3}
"	--	5	47.0×10^{-3}
"	--	10	36.6×10^{-3}

Note: *(delta-A_{419436}/mg total soluble protein)

To express the VHb gene under the control of the tac promoter, an expression plasmid was made using a HindIII-BamHI tac promoter cartridge, the BamHI/SphI fragment from pHbCAT, and the HindIII-SphI digested fragment of the vector pBR322 (Bolivar et al., Construction and characterization of new cloning vehicles. II. A multipurpose cloning system, Gene 2: 95-113, 1977).

With this construct (pINT1), the level of redness of cells correlated well with varying amounts of the gratuitous inducer IPTG, indicating that the gene product synthesis was under the control of tac. The advantages of this expression system are:

a. Higher expression of VHb, and
b. Ability to use complex medium for growth.

EXAMPLE 6

GROWTH OF E. COLI - EXPRESSING VHb
UNDER THE REGULATION OF OTHER PROMOTERS

The aim of this experiment was to demonstrate the growth effects of VHb on E. coli. In thes cases, VHb is expressed using promoters different from the native VHb oxygen-regulated promoter. The strains:plasmids used are:

1. HB101:pBR322 (pBR322 from BRL)
2. JM101:pINT1 (pINT1 discussed in Example 5).

The two hosts have nearly identical genotypes, the major difference being the presence of an F' factor in JM101 which harbors the $lacI^q$ gene. This gene is necessary to keep a strong promoter like tac under control.

The following media recipes shall be henceforth referred to in the appropriate annotated form:

1X LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

2X LB: 20 g/L Bactotryptone, 10 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin

3X LB: 50 g/L Bactotryptone, 25 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 100 mg/L Ampicillin.

The experiment was conducted as follows. Single colonies of the two strains listed above were inoculated into 5 mL 1X LB in a culture tube and grown overnight at 37°C.

0.5 mL of th appropriate inoculum was transferred into 250 mL culture flasks c ntaining 50 mL medium as follows:

1) HB101:pBR322 : 2X LB
2) HB101:pBR322 : 5X LB
3) JM101:pINT1 : 2X LB
4) JM101:pINT1 : 2X LB + 0.1 mM IPTG
5) JM101:pINT1 : 2X LB + 0.5 mM IPTG
6) JM101:pINT1 : 5X LB
7) JM101:pINT1 : 5X LB + 0.1 mM IPTG
8) JM101:pINT1 : 5X LB + 0.5 mM IPTG

Cells were then grown for 24 h at 37°C in a New Brunswick G24 Environmental Incubator Shaker with the shaker speed adjusted to medium setting. At the end of the experiment, the OD_{600} was measured in a Spectronics 21 spectrophotometer by diluting the culture 10-fold in 1% NaCl. The data are listed below.

Host/Plasmid	LB conc.	IPTG conc. (mM)	Final OD_{600}
HB101:pBR322	2X	0	3.00
JM101:pINT1	2X	0	3.03
JM101:pINT1	2X	0.1	2.91
JM101:pINT1	2X	0.5	3.00
HB101:pBR322	5X	0	2.73
JM101:pINT1	5X	0	3.26
JM101:pINT1	5X	0.1	3.40
JM101:pINT1	5X	0.5	3.15

From this data, the following conclusions may be drawn:

1. In all cases involving 2X LB, the cells grew to approximately the same density. This density is roughly twice that obtained routinely in 1X LB

under similar growth conditions and indicates exhaustion of available nutrient. In other words, cells have entered stationary phase of growth due to nutrient limitation.

2. It has b en demonstrat d (Tsai et al., supra) that cells grown in excess nutrient eventually attain an oxygen-limited growth condition due to which they generate inhibitory metabolics such as acetate. Eventually, this leads to cessation of growth, even if more nutrient is supplied. The results of all 5X experiments are indicative of such an occurrence. In other words, oxygen limitation has arisen eventually, causing the culture to reach stationary phase.

3. Hence, it may be argued that under O_2-limited growth, the presence of the hemoglobin gene enhances the growth characteristics of E. coli. This result is similar to that in Examples 3 and 4, with the difference being that here VHb expression is not regulated by DO levels.

4. It appears that under the given growth conditions, there exists an optimal level of VHb expression that maximizes the growth enhancement effect. Such an optimum may be a function of specific growth properties of each cell line and/or plasmid construct as well as of the environmental conditions of growth. The optimum may thus have to be determined for different applications of this technology on a case-by-case basis; however, such determination does not require undue experimentation.

EXAMPLE 7

EFFECT OF VHb PRESENCE ON THE SYNTHESIS OF ANOTHER CLONED PROTEIN IN E. COLI.

The aim of this experiment was to demonstrate the effect of the VHb gene on the synthesis of a model cloned gene product. This is an important application of the technology, since a wide variety

of gene products are produced commercially via recombinant DNA technology. A typical process of this kind involves a high cell density fed-batch fermentation. The productivity of such process s is ultimately limited by insufficient oxygen availability.

The following hosts/plasmids were used in this example:

1. JM101:pCAT
2. JM101:pHbCAT.

The construction of these plasmids is described in Example 5.

The following media compositions were used:

LB: 10 g/L Bactotryptone, 5 g/L Yeast Extract, 5 g/L NaCl, 3 g/L K_2HPO_4, 1 g/L KH_2PO_4, 30 mg/L Chloramphenicol

10X feed: 100 g/L Bactotryptone, 100 g/L Yeast Extract, 150 mg/L Chloramphenicol.

The experiment was conducted as follows:

Single colonies of the two strains were inoculated into 5 ml LB in a culture tube and grown overnight. 1 mL of the inoculum was transferred into 100 mL fresh LB and the growth curve was followed. As cells approached the end of log phase, a 1 mL pulse of 10X feed was added and the growth burst was followed. A second pulse was similarly added. At the end of this-growth phase, a pulse of 1 mL 10X feed containing 100 mM IPTG was added to induce the expression of the CAT gene. One hour later, a sample was withdrawn for monitoring CAT activity. The results of the experiment are shown below.

	JM101:pCAT	JM101:pHbCAT
1) Klett before IPTG pulse	670	700
2) Total soluble protein (mg/ml culture broth)	0.31	0.435
3) CAT activity (units/mg soluble protein)	1.39×10^4	2.67×10^4
4) CAT activity (units/ml culture broth)	4.3×10^3	11.6×10^2

From the above data, the following conclusions may be drawn.

1. The presence of VHb enhances the synthesis of a cloned gene product, even at low levels of VHb industrion.

2. Besides increasing the amount of cloned gene product per unit volume of culture, the presence of VHb may also enhance the specific activity (activity per unit amount of totally soluble protein) of the cloned gene product.

EXAMPLE 8

OXYGEN-DEPENDENT REGULATION OF EXPRESSION OF VHb IN E. COLI BY NATIVE VITREOSCILLA HEMOGLOBIN UPSTREAM SEQUENCES

The aims of this experiment were as follows:

1. To demonstrate that VHb gene expression in E. coli increases in response to decreasing oxygen levels in the medium.

2. To establish transcriptional-level regulation of gene expression.

3. To determine the sensitivity of this oxygen-dependent genetic switch in response to changes in dissolved oxygen concentrations.

Materials and Methods:

The HindIII-SphI fragment containing the VHb gene and flanking sequences was cloned into the corresponding sites of the vector pBR322, thereby creating the plasmid pOX1. This was then transformed into the E. coli hold, HB101. The fermentation was conducted in a New Brunswick Bioflo II fermentor with a 2.5 L working volume using LB (10 g/L Bactotryptone, 5 g/L yeast extract, 5 g/L NaCl, 3 g/L K_2H-PO_4, 1 g/L KH_2PO_4) plus 8 mg/L silicone antifoam as medium at 37°C, pH 7.0 with a constant agitation speed of 300 rpm. All other methods involve conventional protocols (Maniatis, et al., supra).

Cells were grown to an OD_{600} 0.25 with DO maintained greater than 50% air saturation at all times. At this point, the air supply was gradually reduced so that the DO fell to about 1% air saturation in an almost linear manner over a period of 45 min. (i.e., a time scale long enough for gene induction, yet within approximately one generation time of E. coli). Samples were intermittently taken and analyzed for VHb mRNA and protein levels. Later, nitrogen was sparged in the vessel to study the induction of the VHb promoter under strictly anaerobic conditions.

Results:

1. The level of VHb mRNA increased about ten-fold as DO dropped from 70% to 1% air saturation.

2. There was a corresponding increase in VHb activity. A lag was noticed between increase in VHb mRNA level and increase in the quantity of active VHb. This may occur because of the requirement of additional heme biosynthesis in the host cell in order to produce active VHb.

3. The VHb promoter was switched on to significant levels only below 40% air saturation and

attains maximum induction levels below 5% air saturation.

4. The promoter switches off under strictly anaerobic conditions, indicating the importance of a basal level of aerobicity in the environment for maximal gene expression.

EXAMPLE 9

OXYGEN-DEPENDENT EXPRESSION OF ANOTHER CLONED PROTEIN

In order to use the VHb oxygen-regulated promoter element (ORE) to express other genes, deletions were made from the cloned Vitreoscilla fragment described in Examples 1 and 2 to isolate a functional promoter element. The enzyme Bal 31 was employed for this purpose (Maniatis, et al., supra). One of the fragments isolated extended from the 5' end of the sequence listed in Example 2 to approximately 125 bp downstream, as sized on a 6% polyacrylamide gel. This fragment was used to express a gene different from that for VHb under oxygen-dependent regulation.

In this experiment, the fragment just described was fused to a promoterless chloramphenicol acetyl transferase (CAT) gene cartridge purchased from Pharmacia Inc. This fusion was inserted into the HindIII-SphI sites of the vector pBR322 to create the plasmid, pOX1. This was transformed into the E. coli host, HB101.

To test the functionality of the ORE, an experiment similar to that in Example 8 was conducted. CAT gene product assays were conducted using a conventional protocol (Neumann, et al., supra).

S. cerevisiae strain 488-0 (leu2, ura3, his 1-7) was transformed with plasmids AAH5 and pEX-2 by the rapid colony transformation procedure (Keszenman-Pereyra and Heida, A colony procedure for transformation of Saccharomyces cerevisiae, Curr. Genet. 13: 21-23, 1988), and plated on synthetic dextrose (SD) medium (Rose, Isolation of genes by complementation in yeast, Methods in Enzymology, 152: 481-504, 1987) without leucine. A representative clonal cell line from each transformation was established after colony purification of a primary transformant.

For the growth studies, single yeast colonies were inoculated into 2 mL of SD -leu (+leu for 488-0) and cultured for 24 hr at 260 rpm at 30°C in a Labline Model 3258 Orbit Enviro-shaker. 0.5 mL of this inoculum was added to 50 mL of the same medium in a 250 mL flask and cultured at 260 rpm at 30°C. Cell growth was measured by turbidity (A_{600nm}) using a Perkin-Elmer Lambda 4A Spectrophotometer. When the glucose level of the culture medium dropped below 2.0 mM, the cultures were pulsed with 1/40 volume of a concentrated medium containing 20 x SD (40% glucose, 13.3% Difco yeast nitrogen base without amino acids, and 1.6 mg/mL of all the amino acids except leucine. For strain 488-0, 1.6 mg/mL leucine was included in the pulse medium) Glucose concentration was estimated using Ames Glucostix test strips.

Results:

A comparison of the growth curves of strains 488-0, 488-0:AAH5, and 488-0:pEX-2 grown under the above conditions revealed the following:

1. All three strains grew at an equivalent rate during the logarithmic stage of growth.

Results:

At DO = 70% air saturation, the CAT activity in E. coli HB101:pOX1 was $6.1x10^4$ units/mg soluble prot in. After maintaining the DO between 2 to 5% air saturation for 45 min., CAT activity in E. coli HB101:pOX1 was $6.3x10^5$ units/mg soluble protein. This demonstrates the isolation of a functional ORE capable of expressing proteins other than Vitreoscilla hemoglobin under control of dissolved oxygen content of the culture.

EXAMPLE 10

GROWTH ENHANCEMENT OF SACCHAROMYCES CEREVISIAE CELLS EXPRESSING VHb

In this example, the effect of VHb expression on the growth of the yeast Saccharomyces cerevisiae was studied. The VHb gene was cloned into a yeast expression plasmid, AAH5, that is stably maintained as an extrachromosomal plasmid in yeast cells.

Materials and Methods:

Plasmid pEX-2 was constructed as follows. The BamHI/SphI fragment described in Example 5 was cloned by blunt-end ligation into the HindiII site of the yeast expression vector AAH5 (Ammerer, Expression of genes in yeast using the ADC-1 promoter, Methods in Enzymology 101: 192-201, 1983). AAH5 contains the selectable yeast marker Leu 2, the 2 micron circle origin of replication, and a unique HindIII site flanked by the transcriptional promoter and terminator regions of the yeast alcohol dehydrogenase-1 (ADH-1) gene. The ADH-1 promoter will support high levels of transcription of any sequence cloned into the HindIII site. The ADH-1 gene is constitutively expressed in yeast.

2. The VHb-containing strain 488-0:pEX-2 grew to a final optical density of 13.0, while strains 488-0:AAH5 and 488-0 grew to optical densities of only 10.0 and 9.5, respectively. This represents a 26.0% increase in final cell density b tween a strain carrying the VHb gene on a plasmid (488-0:pEX-2) compared to a strain containing the identical plasmid without the VHb gene (488-0:AAH5). In addition, this represents a 32.6% increase in the final cell density of 488-0:pEX-2 over the strain containing no AAH5-derived plasmid (488-0).

EXAMPLE 11

GROWTH ENHANCEMENT DUE TO EXPRESSION OF VHb IN E. COLI FROM A CHROMOSOMALLY INTEGRATED GENE

In this example, the tac-VHb gene fusion, discussed above, was integrated into the chromosome of E. coli MG1655 (obtained from Cold Spring Harbor Laboratory, NY).

Materials and Methods:

A defective Tn10 transposon (Foster, et al., Three Tn10-associated excision events: Relationship to transposition and role of direct and inverted repeats, Cell, 23: 215-227, 1981) was constructed as follows. A kanamycin resistance gene (Pharmacia Inc.) was cloned into the SalI site of pINT1 (Example 5). The EcoRI/EagI fragment from the resulting plasmid, which contains the entire tac-VHb fusion and Kan^R gene, was cloned between the inverted repeats (bases 1-66 on the right end and bases 9234-9300 on the left end) of a Tn10 derivative which lacks the transposase gene (obtained from Cold Spring Harbor Laboratory, NY). The resulting element, Tn10dKan-tac-VHb, was cloned into a multicopy plasmid containing a tac-Tn10 rightward transposase (obtained

from Cold Spring Harbor Laboratory, NY). Transposition was induced with 0.5 mM IPTG for 4 hr, f llowing which cells were plated on lactose-MacConk y-Kan plates. Lac mutants were selected and the transposon-induced mutation was induced into E. coli MG1655 using P1 phage (Silhavy, et al., supra). One of the resulting Lac$^-$ colonies, which was further purified and checked for Lac$^-$Kan^R, Amp^S, VHb$^+$ (IPTG inducible, as confirmed by assay described in Example 3), was designated GRO13. Comparison of growth properties of strains MG1655 and GRO13 in 2X LB (described in Example 6) containing 1 mM IPTG, followed by addition of a concentrated feed (25% Bactotryptone, 12.5% yeast extract), showed an increase in final cell densities (final cell densities: OD_{600} = 16.8 for MG1655, OD_{600} = 18.1 for GRO13).

EXAMPLE 12

Oxygen-regulated Expression of Vitreoscilla hemoglobin from a single-copy, integrated hemoglobin gene.

Two strains of E. coli were developed that contain the Vitreoscilla hemoglobin gene and its promoter, integrated into the chromosome. The hemoglobin gene region was inserted into the chromosome of wild type strain MG1655 (obtained from the Cold Spring Harbor Laboratories) via transposon-mediated integration. The two strains, GRO21 and GRO22, have the hemoglobin gene region inserted into the host lac and xyl operons, respectively.

In order to determine the extent of oxygen-dependent regulation of the integrated hemoglobin expression was monitored during growth in a shake-flask. The cells were grown in 100 ML of a buffered complex

media [10 g/L yeast extract, 5 g/L yeast extract, g/L NaCl, 3 g/L K_2HPO_4, a g/L KH_2PO_4 (pH 7)] at 200 rpm in a New Brunswick G24 shaker-incubator at 37°C. The larg culture volume ensured that the culture became xygen-limited before any other nutrient became limiting or before significant levels of organic acids had accumulated.

Hemoglobin expression was determined by Western analysis using anti-hemoglobin antiserum. In both strains, hemoglobin was not detectable in early exponential-phase cells when the culture oxygen concentration was high. Later, when oxygen became limited (OD_{590}=0.9), hemoglobin levels increased significantly. At an OD_{590} of 2.8, hemoglobin expression decreased, indicating that hemoglobin gene promoter activity is lower when the oxygen concentration is reduced to near-zero levels.

Stability of the integrated genes was confirmed by subculturing the cells and demonstrating hemoglobin expression after twenty generations of growth.

In summary, significant amounts of hemoglobin were expressed from a single, integrated copy of the hemoglobin gene. Activity of the integrated gene was regulated by oxygen and maximal expression was achieved under microaerobic conditions. This is similar to its expression properties on a multicopy plasmid.

EXAMPLE 13

The Vitreoscilla Hemoglobin Gene Promoter Region Contains Two Major Transcriptional Initiation Sites

Primer extension analysis was employed to identify transcriptional start site(s) (promoters) within the upstream region of the Vitreoscilla hemoglobin gene. In order to obtain higher levels of hemoglobin RNA, the Vitreoscilla hemoglobin gene region [Khosla and Bailey (1988) Mol. Gen. Genet., 214: 158] was cloned into the multicopy plasmid pBR322. The resultant plasmid, pOX1, was then introduced into the E. coli host strain HB101 (F⁻hsdS20 recA13 ara14 proA2 lacY1 galK2 rpsL20 xyl5 mtl1 supE44).

A 30-mer oligonucleotide corresponding to bases +32 to +1 (relative to the translation initiation codon) of the hemoglobin gene was hybridized to total messenger RNA (mRNA) isolated from HB101pOX1 cells. The oligo/RNA hybrids were extended with reverse transcriptase and radiolabeled trinucleotides [Kingston (1978) in Current Protocols in Molecular Biology, Ausubel, et al., eds., John Wiley, 4.8.1] and the products resolved on an acrylamide/urea sequencing gel. Two major extension products were visualized. The most intense band mapped to position -56 which is located 10 bp downstream of a putative Pribnow box. The less intense band mapped to position -109. This result indicated that there are two major transcription initiation sites within the hemoglobin gene promoter. The identical experiment using mRNA from cells growing at different oxygen concentrations indicated that both promoters are oxygen-regulated and activated under microaerobic conditions.

EXAMPLE 14

The Cyclic AMP-Catabolite Activator Protein (cAMP-CAP) Complex is Involved in the Regulation of the Vitreoscilla Hemoglobin Gene Promoter in Escherichia coli

A plasmid, pOX11, was constructed to study the regulatory properties of the Vitreoscilla hemoglobin promoter in E. coli. pOX11 was constructed by fusing the upstream region and first 11 codons of the Vitreoscilla hemoglobin gene [Khosla and Bailey (1988), Mol. Gen. Genet., 214: 158], to the E. coli lacZ gene [Berman, et al., Gene Anal. Tech., 1: 43]. The lacZ gene contained a small deletion of 5' coding sequence, but retained the ability to synthesize active B-galactosidase (B-gal). This fusion was inserted into the multicopy E. coli plasmid pBR322. B-gal activity was used as the indicator of hemoglobin gene promoter activity.

To test whether the cAMP-CAP system was involved in the overall control of the promoter, pOX11 was transformed into a control E. coli strain MC4100 [araD139delta (argF-lac)U169 rpsL150 relA1 flbB5301 ptsF25 deoC1, Casadaban (1986), J. Mol. Biol., 104: 541] and two derivatives of MC4100 and two E. coli strains that are unable to synthesize either cAMP [GE1051 (delta cya 854 ilv::Tn10] or CAP [GE1050 (delta crp cam)]. Strains GE1051 and GE1050 were obtained from Dr. G. E. Weinstock (University of Texas). In order to achieve maximum induction of the promoter, the cells were grown in 250 mL shake-flasks containing 100 mL of buffered complex medium (described in Example 12) at medium setting in a New Brunswick G24 shaker-incubator.

Specific B-gal activity was measured in the three strains throughout the growth curve. The maximum B-gal 1 vel observed in the control strain MC4100:pOX11 was above 5000 Miller Units, representing a 10-fold increase over background levels. In contrast, the maximum B-gal levels observed in GE1051:pOX11 were less than 500 Miller units, representing less than a 3-fold increase over background. Addition of cAMP to the GE1051 (cya$^-$):pOX11 culture restored the 10-fold indication seen in the control strain. In addition, nucleotide sequence analysis has identified a consensus CAP binding site at position -91 to -101 (with respect to the translation initiation codon) in the Vitreoscilla hemoglobin gene region. These results indicate that the cAMP-CAP complex is involved in the overall regulation of the hemoglobin gene promoter.

EXAMPLE 15

Activation of the Vitreoscilla Hemoglobin Gene Promoter in a Fed-Batch Fermentation

Another plasmid, pOX2, was constructed to study the regulatory properties of the Vitreoscilla hemoglobin gene promoter in E. coli. The plasmid pOX2 was constructed by fusing the promoter to the chloramphenicol acetyltransferase (CAT) gene (obtained from Pharmacia). This operon fusion was inserted into the BamHI/HindIII site of the E. coli multicopy plasmid pBR322 to give pOX2. CAT activity [Neuman, et al. (1987), Biotechniques, 5:444] was used as the indicator of promoter activity.

The promoter was introduced into E. coli wild-type strain MG1655 (see Example 12). The promoter activity was monitored throughout a fed-batch growth experiment where the culture dissolved oxygen (DO)

was initially kept high and then rapidly dropped. To initiate the xperiment, 2.5 mL of a 12-hour seed culture was inoculat d into 2.5 L of a buffered c mplex m dium [2% yeast extract, 0.5% glycerol, 0.3% K_2HPO_4, 0.1% KH_2PO_4, 0.1% trace metal mix (8.3 mM Na_2MoO_4, 7.6 mM $CuSO_4$, 8 mM H_3BO_3), 0.1% vitamin mix (0.042% riboflavin, 0.54% pantothenic acid, 0.6% niacin, 0.14% pyridoxine, 0.006% biotin, 0.004% folic acid), 1 mM MgSO4, 0.05 mM $CaCl_2$, 0.2mM $FeCl_3$, 50 µg/mL ampicillin, pH 7) in a New Brunswick Bioflo III fermentor. The air flow rate was kept constant at 4L/minute. The DO was maintained above 50% of air saturation by increasing the agitation rate as the cells grew. When the culture reached an OD_{590} of 2.5, concentrated feed medium [43% glycerol, 11% $(NH_4)_2SO_4$, 0.8% $MgSO_4$, 1% trace metal mix, 1% vitamin mix, 0.2 mM $FeCl_3$, 0.05 mM $CaCl_2$, and 40 µg/ml ampicillin] and 50% yeast extract (added separately) were added at a rate of 3 mL/hour. The feed rates were progressively increased to maintain exponential growth until the culture reached on OD_{590} of 12.0. During this time, the specific CAT activity increased from 2.1 units/mL-OD_{590} to 5.9 units/mL-OD_{590}, indicating a 3-fold induction of the promoter occurred at high DO levels.

When the cells reached an OD_{590} of 12.0, the air supply was reduced to 3 L/minute and the agitation lowered to 500 rpm. Under these conditions, the culture DO dropped to 0-1% air saturation within one minute. Within 45 minutes, the CAT activity increased to a maximum of 64.3 units/mL-OD_{590}. This indicated an 11-fold induction of the promoter occurred by reducing the DO.

These results indicate that a reduction in culture oxygen concentration can substantially activate the Vitreoscilla hem globin gene promoter in a fed-batch fermentation. In addition, there is at least one oth r regulator of promoter activity besides oxygen concentration. In this example, a greater than 30-fold overall induction of CAT was observed.

EXAMPLE 16

The Early, Oxygen-Independent Induction of the Vitreoscilla Hemoglobin Gene Promoter is Suppressed by High Levels of Yeast Extract

The experiment in Example 15 indicated that there is at least one additional mode of the promoter regulation other than oxygen concentration. This regulation was apparent in exponential-phase cells when the DO was kept above 50% air saturation in a fed-batch fermentation. To address whether a culture medium component was involved in the oxygen-independent regulation, MG1655:pOX2 cells were grown in a fed-batch fermentation with different concentrations of yeast extract. The batch and feed media were the same as in Example 15, except that either 0.1%, 0.5%, or 2.0% (w/v) yeast extract was used. There was no yeast extract in the feed medium. The cells were grown in batch mode until an OD_{590} of approximately 2.5. Feed medium was then introduced at a rate of 2.5 mL/hour. By adjusting the air flow and agitation rates, the DO was maintained above 50% air saturation for the entire experiment. CAT activity was monitored throughout the experiment. The maximum values as follows:

Yeast Extract (%)	OD590	CAT (units/mL-OD590)
0.1%	4.0	11432
0.5%	4.0	4544
2.0%	4.0	1606

These results indicate that the early oxygen-independent induction of the Vitreoscilla hemoglobin gene promoter is suppressed by high levels of yeast extract.

EXAMPLE 17

Enhancement of Cloned Chloramphenicol Acetyltransferase Production in a Fed-Batch Fermentation Using E. Coli Cells Expressing Hemoglobin from a Single-Copy, Integrated gene

A plasmid containing the chloramphenicol acetyltransferase (CAT) gene was introduced into E. coli strains GRO22 and MG1655 (see Example 12) in order to examine the effect of hemoglobin expression on the production of a cloned protein. The plasmid, pTCAT, was constructed by inserting the CAT gene (obtained from Pharmacia) into the multicopy plasmid pKK223-3 (obtained from Pharmacia) which places the CAT gene under control of the tac (trp-lac) promoter. The tac promoter is constitutively active in MG1655.

The cells were grown in a 2.5 L fermentor in a buffered defined medium containing 0.5% glucose, 0.15% KH_2PO_4, 0.43% K_2HPO4, 0.04% $(NH_4)_2SO_4$, 0.05 mM $CaCl_2$, 0.2 mM $FeCl_3$, 1 mM $MgSO_4$, 0.1% trace metals (Example 15), 0.1% vitamins (Example 15), and 50 µg/mL ampicillin. The agitation speed was maintained at 300 rpm and the air flow rate maintained at 0.2 L/minute. When the culture reached an OD_{590} of 1.2, feed medium (Example 15, except that 43% glucose was used instead of glycerol) was added at a rate of 1.4 mL/hour. The feed rate was increased to 2.8 mL/hour when the culture reached an OD_{590} of 2.2.

CAT activity was monitored throughout the growth period. The results are shown in the following Table:

Strain	Time (h)	CAT Activity (units/mL)
MG1655:pTCAT	10	330
MG1655:pTCAT	12	380
MG1655:pTCAT	14	440
MG1655:pTCAT	15	480
MG1655:pTCAT	21	430
GRO22:pTCAT	11	420
GRO22:pTCAT	14	740
GRO22:pTCAT	15	820
GRO22:pTCAT	16	770

As can be seen, there is a significant enhancement of CAT production in GRO22:pTCAT culminating with a 71% increase at 15 hours. In addition, there was an approximately 10% increase in total cell protein in GRO22:pTCAT cells relative to total cell protein in MG1655:pTCAT. Western analysis using an anti-CAT antibody indicated that CAT expression was equivalent in the two strains until they reached oxygen limitation (approximately 6 hours). This experiment indicates that an E. coli strain expressing hemoglobin from a single copy, integrated gene can be used to produce significantly higher quantities of a cloned protein. In addition, product enhancement with this system occurs when the culture oxygen concentration is low.

IN THE CLAIMS:

1. A method for preparing any foreign protein in a host cultured cell which grows within a first level f environmental oxygen comprising:

(a) introducing into said host cultured cell a DNA expression vector capable of replicating in said host cultured cell, said vector having a promoter/regulator capable of directing transcription and translation initiation and control of foreign recombinant-DNA sequences for coding for said foreign protein located downstream therefrom, said promoter/regulator being derived from an organism other than said host, and said promoter/regulator further being capable of being activated upon a decrease of environmental oxygen to below said first level;

(b) culturing said host cultured cell in a medium appropriate for expressing the protein expressed by said foreign DNA; and

(c) lowering the level of oxygen available to said medium to a second level below said first level, said second level providing an oxygen concentration which activates said promoter/regulator.

2. A method for preparing any foreign protein in a host cultured cell which grows within a first level of environmental oxygen comprising:

(a) introducing into said host cultured cell a DNA expression vector comprising a series of nucleotides including the Vitreoscilla hemoglobin transcription and/or translation initiation sequences capable of directing intracellular production of a major portion of the Vitreoscilla hemoglobin protein, said vector containing foreign DNA sequences coding for said foreign protein, said vector having a promoter/regulator capable of being activated by a

d crease of environmental oxygen to below said first level;

(b) growing said host cultured cell in a medium appropriate for isolating the protein expressed by said foreign DNA; and

(c) lowering the level of oxygen available to said medium to a second level below said first level, said second level providing an oxygen concentration which activates said promoter/ regulator.

3. The method of Claim 2 wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

4. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing said host cultured cell to produce a protein having at least some oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein; and

(d) permitting the protein to assume an active structure whereby it possesses at least some oxygen-binding activity.

5. The method of Claim 4 wherein said portable DNA sequence is introduced directly and int grated into th chromosome of a host cultured cell.

6. The method of Claim 4, wherein said portable DNA sequence is introduced into said host cultured cell by the following steps:

(a) cloning the portable DNA sequence into a vector capable of being transferred into and replicating in a host cell, such vector containing operational elements for the portable DNA sequence;

(b) transferring the vector containing the portable DNA sequence and operational elements into a host cultured cell capable of expressing at least some of the oxygen-binding protein; and

(c) culturing the host cell under conditions appropriate for replication and propagation of the vector and expression of the protein.

7. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering, biopolymers, and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein; and

(d) permitting the protein to assume an active structure whereby it possesses at least some oxyg n-binding activity.

8. A method for transporting and supplying oxygen to oxygen-requiring processes and operations, comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein;

(d) permitting the protein to assume an active structure whereby it possesses at least some oxygen-binding activity; and

(e) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-requiring process.

9. A method for the binding and removal of oxygen form an environment comprising:

(a) preparing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity;

(b) introducing said portable DNA sequence into a host cultured cell capable of expressing at least some oxygen-binding protein;

(c) culturing the host cell under conditions appropriate for expression of the protein;

(d) permitting the protein to assume an active, structure whereby it possesses at least some oxygen-binding activity; and

( ) effectively delivering said host cell or a preparation from said host cell containing the oxygen-binding protein to said oxygen-containing environment.

10. A method for increasing the growth characteristics, including the growth yield, the growth rate, and the achievable cell density under controlled circumstances, of a cell in culture, said host cultured cell being chosen and derivatized from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants, and insects, said cell being capable of producing a protein having at least some oxygen-binding activity, comprising:

(a) culturing said host cell under conditions appropriate for expression of said protein, and

(b) permitting said protein to assume an active structure whereby it possesses at least some oxygen-binding activity.

11. A method for increasing the production of proteins, both those normally made and those expressed as a result of genetic engineering, biopolymers, and other metabolic products, of a host cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells consisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects, said cell being capable of producing a protein having at least some oxygen-binding activity, comprising:

(a) culturing said host cell under conditions appropriate for expression of said protein, and

(b) permitting said pr tein to assume an active structure whereby it possesses at least some oxygen-binding activity.

12. A method according to any one of the Claims 1 through 30 further comprising the step of modulating the activity of said promoter/regulator by control of the amount of cAMP-CAP complex in said host cell.

13. A method according to Claim 12 wherein said step of modulating said activity comprises introducing cAMP into said host cell, wherein said host cell is unable to synthesize CAP or cAMP.

14. A method according to Claim 12 wherein said step of modulating said activity comprises manipulation of the activity of a crp gene in said host.

15. A method according to any one of Claims 4 through 11 further comprising the step of modulating the expression of said protein by control of the amount of cAMP-CAP complex in said host cell.

16. A method according to Claim 15 wherein said step of modulating said expression comprises introducing cAMP into said host cell, wherein said host cell is unable to synthesize CAP or cAMP.

17. A method according to Claim 16 wherein said step of modulating said expression comprises manipulation of the activity of a crp gene in said host.

18. A method for regulating the production of at least one designated protein, selected from the group consisting of those proteins normally made and those expressed as a result of genetic engineering, biopolymers, and other metabolic products, of a host

cultured cell grown in the presence of oxygen, said host cultured cell being chosen and derived from a selection of cultured cells c nsisting of microorganisms and cells obtained from multi-cellular organisms selected from the group consisting of animals, plants and insects comprising:

(a) introducing a portable DNA sequence capable of directing a host cultured cell to produce a protein having oxygen-binding activity into a host cultured cell;

(b) culturing said host cell under conditions appropriate for expression of said oxygen-binding protein and said designated protein.

19. A method according to Claim 18 wherein said designated protein is expressed from an extrachromosomal element in said cell.

20. A method according to Claim 19 wherein said oxygen-binding protein comprises Vitreoscilla hemoglobin.

21. A method according to Claim 20 wherein said designated protein is chloramphenicol acetyltransferase.

22. A method for preparing any foreign protein in a host cultured cell which grows within a first level of environmental oxygen comprising:

(a) introducing into said host cultured cell a DNA expression vector capable of replicating in said host cultured cell, said vector having a promoter/regulator capable of directing transcription and translation initiation and control of foreign recombinant-DNA sequences coding for said foreign protein located downstream therefrom, said promoter/regulator being derived from an organism

other than said host, and said promoter/regulator being capable of being activated upon a decrease of environmental oxygen to below said first level and further capable of being suppressed ind pendently of environmental oxygen concentration during exponential growth of said host cultured cell;

(b) culturing said host cultured cell in a suitable medium appropriate for expression of said foreign DNA; and

(c) introducing a source of complex nitrogen into said medium during exponential growth of said host to a level sufficient to suppress said promoter/regulator.

23. A method of preparing any foreign protein in a host cultured cell which grows within a first level of environmental oxygen comprising:

(a) introducing into said host cultured cell a DNA expression vector comprising a portable DNA sequence comprising a series of nucleotides capable of directing intracellular production of a major portion of the Vitreoscilla hemoglobin protein, said vector containing foreign DNA sequences coding for said foreign protein, said vector having a promoter/regulator capable of being activated by a decrease of environmental oxygen to below said first level and capable of being suppressed independently of environmental oxygen concentration during exponential growth of said host cultured cell;

(b) growing said host cultured cell in a medium appropriate for expression of said foreign DNA; and

(c) introducing a source of complex nitrogen into said medium during exponential growth of said host to a level sufficient to suppress said promoter/regulator.

24. The method of Claim 23, wherein said portable DNA sequence is introduced directly and integrated into the chromosome of a host cultured cell.

25. The method of Claim 23 wherein said portable DNA sequence comprises the Vitreoscilla hemoglobin transcription and/or translation initiation sequences.

26. The method according to Claim 22 wherein said source of complex nitrogen comprises yeast extract.

27. The method according to Claim 23 wherein said source of complex nitrogen comprises yeast extract.

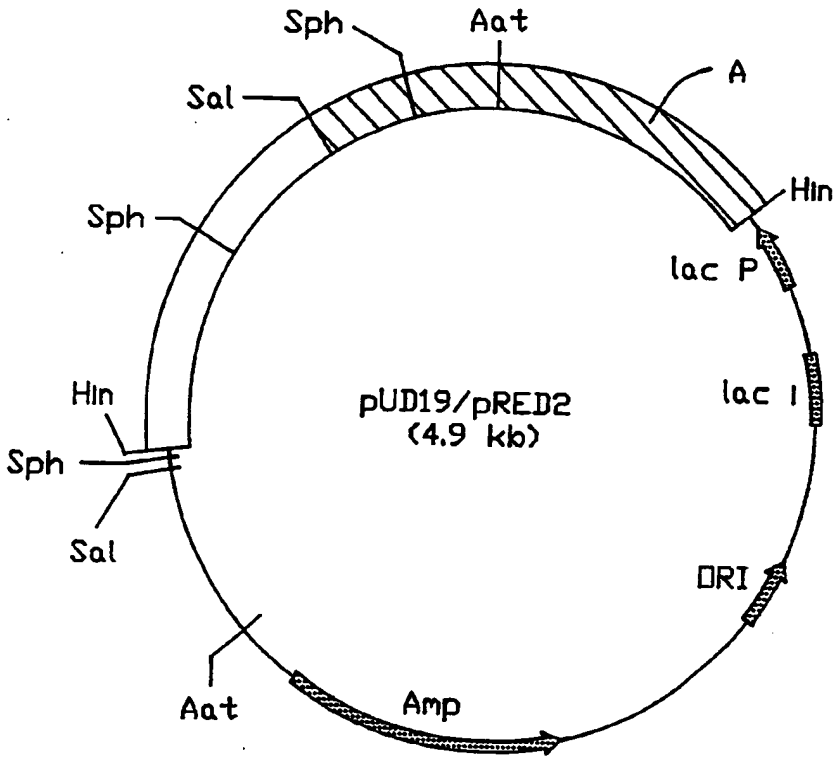

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FIG.-1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/06083

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C12N 15/00, 15/31, 1/00; A61L 9/01

U.S.Cl: 435/69.1, 172.3, 317.1, 266

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S. Cl.	435/69.1, 172.3, 317.1, 266, 42, 801, 818

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

See Attachment

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
X Y	WO, A, 89/03883 (Khosla et al.) 05 May 1989, see claims 44-58.	1-11,18-21 12-17,22-27
X Y	BioTechniques, Volume 7, Number 9, issued 25 October 1989, Hughes et al, "A New Oxygen-Regulated Promoter for the Expression of Proteins in Escherichia coli", pages 1026-1028. See entire document.	1,2 12-17,22-27
Y	F. Neidhardt et al., "Escherichia coli and Salmonella typhimurium", published 1987 by American Society for Microbiology (Washington, D.C.), see pages 1318-1325, especially pages 1319-1321.	12-17
X	Molecular and General Genetics, volume 214, issued 1988, Khosla et al, "The Vitreoscilla hemoglobin gene: Molecular cloning, nucleotide sequence and genetic expression in Escherichia coli", pages 158-161. See entire document.	4,4,6,7,10,11 18-20

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
14 January 1991	06 FEB 1991
International Searching Authority [1]	**Signature of Authorized Officer** [20]
ISA/US	Mary E. Mosher

Form PCT/ISA/210 (second sheet) (May 1986)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No [18]
X	Nature, volume 331, issued 18 February 1988, Khosla et al, "Heterologous expression of a bacterial haemoglobin improves the growth properties of recombinant Escherichia coli", pages 633-635. See entire document.	4,6,7,10,11 18-20
X,P	Journal of Bacteriology, volume 171, No. 11, issued November 1989, Khosla et al, "Characterization of the Oxygen-Dependent Promoter of the Vitreoscilla Hemoglobin Gene in Escherichia coli", pages 5995-6004. See entire document.	1-3,12-17
X,P Y,P	Nucleic Acids Research, volume 18, number 14, issued 25 July 1990, Dikshit et al, "Study of Vitreoscilla globin (vgb) gene expression and promoter activity in E. coli through transcriptional fusion", pages 4149-4155. See entire document.	1,2,4,6,7, 10,11,18-27 12-17

FURTHER INFORMATION C NTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This International search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment to Telephone practice

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application. Telephone practice

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Attachment To PCT/IPEA/210
USPTO Automated Patent System (file USPAT, 1970-1991), CAS.

Search terms:

Vitreoscilla, hemoglobin, globin, artificial blood, oxygen transport; author search Khosla, C; Bailey, J; Dikshit, K; Webster, D.